DIVALENT CATION UPTAKE IN THE YEAST, *CANDIDA UTILIS*

Thesis submitted for the degree of Doctor of Philosophy

1987

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ABSTRACT

Toxicity of Cu^{2+} and Mn^{2+} became apparent at 10 μM and 40 mM respectively, as determined by viable plate counts and respirometry. Mn^{2+} and Cu^{2+} accumulation from non-toxic concentrations comprised an initial energy-independent binding to external cellular surfaces followed by metabolism-dependent influx which was blocked by low temperature, the absence of an energy source and by a number of metabolic inhibitors.

Competition studies revealed the presence of three separate transport mechanisms; a non-specific divalent cation uptake system (putative primary Mg^{2+}-carrier), a Mn^{2+}-specific transporter and a Cu^{2+}-specific transporter. Mn^{2+} uptake *via* the non-specific uptake system was a saturable process giving a K_t of 65.3 μM. Uptake was competitively inhibited by Mg^{2+}, Co^{2+} and Zn^{2+} giving K_i values of 64 μM, 100 μM and 184 μM respectively.

Mn^{2+} uptake from nanomolar Mn^{2+} concentrations was *via* the Mn^{2+}-specific carrier and was unaffected by 100-fold molar excess of Mg^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, Ni and Cu^{2+}. Analysis of the uptake kinetics revealed a K_t of 16.4 nM. Zn^{2+} competitively inhibited Mn^{2+}-specific uptake, the K_i being 500-fold greater than the K_t for Mn^{2+}. Cu^{2+}-specific uptake was unaffected by levels of Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} 10-fold that of Cu^{2+} and gave a K_t of 3.1 μM.

Efflux studies indicated a small metabolic exchange of Mn^{2+}. Mn^{2+} influx was balanced by a stoichiometric loss of protons from the cell. All three transport systems were highly pH-dependent. Cu^{2+} and Mn^{2+} uptake was reduced by high external K^+. Phosphate

stimulated Mn^{2+} uptake via the non-specific carrier. By varying the concentrations of their respective substrates in the growth media, both the non-specific divalent cation transporter and the Mn^{2+}-specific transporter were shown to be inducible-repressible systems. The Cu^{2+} uptake system was constitutive. Cellular Mn^{2+} remained constant during growth in batch culture. These specific systems appear analogous to the specific metal transport systems reported in bacteria and may be widespread in microorganisms.

ACKNOWLEDGEMENTS

I would like to extend my warmest thanks to my supervisor, Dr.I.S.Ross, Department of Biological Sciences, University of Keele, for his continued encouragement and support throughout this study.

Gratitude is also extended to all those members of the Department of Biological Sciences, University of Keele, who provided assistance during my studies, in particular Dr.J.Mills, for his advice and practical help performing surface charge density measurements.

The Science and Engineering Research Council is gratefully acknowledged for the receipt of a research studentship which made this study possible.

PUBLICATIONS

The following reports on aspects of this study have been previously published and are included in Appendix II:

1. Parkin, M.J. & Ross, I.S. (1985)
   Uptake of copper and manganese in the yeast, *Candida utilis*.
   Microbios Letters **29** 115 - 120.

2. Parkin, M.J. & Ross, I.S. (1985)
   The transport and accumulation of manganese in the yeast,
   Candida utilis. In: *Heavy Metals in the Environment*, Athens,
   Vol 2, pp. 289 - 291, Ed. T.D. Lekkas, CPC Consultants, Edinburgh.

3. Parkin, M.J. & Ross, I.S. (1986)
   The specific uptake of manganese in the yeast, *Candida utilis*.
   Journal of General Microbiology **132** 2155 - 2160.

4. Parkin, M.J. & Ross, I.S. (1986)
   The regulation of Mn^{2+} and Cu^{2+} uptake in cells of the yeast
   Candida utilis grown in continuous culture.
   FEMS Microbiology Letters **37** 59 - 62.

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1. GENERAL INTRODUCTION

Microbes play an intrinsic role in the biogeochemical cycling of elements in the biosphere. Interactions between organisms and mineral elements in the environment occur at a number of levels. Metabolic processes may result in the accumulation of essential metals or may effect biochemical transformations, bringing about changes in the valence of the metal or resulting in phenomena such as biomethylation or alkylation (Jernelov & Martin, 1975). In addition, mobilisation of metal ions in the ecosystem may occur following microbial metabolism and growth and subsequent alterations in environmental pH, redox potential and organic content. Of these, the ability of microorganisms to accumulate certain inorganic ions is of importance when considering the elementary composition of cells and in determining which elements are essential to cells.

The major covalently-bound elements, C, O, N, H, S and P, collectively constitute about 99 % of cellular dry weight and their essential functions in living cells are well understood (Aiking, 1977). However the trace elements are of particular interest as, despite much work in this field, comparatively little is known of their role, cellular concentration and mode of entry into cells. The primary objective of this study was to examine the uptake of essential divalent cations, normally present in the external milieu at trace concentrations, in the yeast *Candida utilis*.

Interactions between microorganisms and metals have been widely studied in the past. Some early studies, in particular of Cu^{2+} and Hg^{2+}, were initiated as a result of the value of these metals as fungicides and the inhibition of fungal spore germination by heavy

metal ions has received much attention (Somers, 1963). The development of strains of fungi resistant to heavy metals stimulated an interest into the mechanisms of adaptation and several useful reviews have appeared (Ashida, 1965, Ross, 1975). Consequently, the need to develop some understanding of the basis for metal resistance led to studies of accumulation and metabolism of these metals being undertaken, and these have contributed considerably to our current knowledge of microbial metal transport.

According to Wood & Wang (1983), huge changes in the distribution and solubilisation of metal ions in the environment have occurred over the past two centuries, resulting from industrial activities such as mining, metal-working and chemical production. The release of metal pollutants, many of which are toxic, and their subsequent effect on microbial ecology has been cause for concern. Metal uptake by microorganisms may lead to their incorporation into the food chain and ultimately the elements may be absorbed by higher animals or man (Broda, 1972). The ability of yeasts to accumulate metal ions is clearly demonstrated in a recent report of Cu^{2+} accumulation by yeast biomass during single cell protein production (Quinn *et al*, 1981). The feedstock for this process was distillery spent wash, which contains relatively high levels of Cu^{2+}, and it was observed that growth of a mixed culture of *Geotrichum candidum*, *Candida kruzei* and *Hansenula anomala* led to the accumulation of Cu^{2+} in the biomass to levels generally in excess of those recommended for most animal diets, other than pigs. As stillage from various sources used in biomass production may commonly contain elevated metal concentrations (Sheehan & Greenfield, 1980), the accumulation of heavy metals by fodder yeasts may be of considerable importance.

Recently, due to the growing scarcity and increasing value of some metals, and to a greater awareness of the ecological effects of toxic metals, the possibility of using microorganisms for the removal or recovery of metals from dilute solutions has been considered. Over the last decade a number of international conventions have agreed on legislation to limit the discharge of metals into the aquatic environment. One outcome of these directives is that stricter statutory limits are likely to be imposed in the near future, rendering many conventional metal recovery techniques inadequate (Townsley *et al*, 1986). Hence, it now appears that microbes may have some potential for commercial and industrial metal removal whereas a system of this nature was previously considered to be uneconomic. The availability of yeasts as by-products from large-scale commercial processes, together with their use in numerous reports of metal uptake, make them suitable organisms with which to study the applicability of microorganisms as metal removal agents (Norris & Kelly, 1979).

Improvements in the development and sensitivity of analytical techniques, such as atomic absorption spectroscopy, anodic stripping voltammetry and X-ray microprobe analysis, coupled with the wider availability and use of radioactive nuclides has greatly facilitated the recent surge of interest in microbial metal uptake. This has led to some important advances in our understanding of metal transport systems in bacteria, yeasts and fungi which have been of considerable academic interest.

A number of metal ions are essential for optimal growth of yeasts and fungi and these include Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} ions. The role played by these inorganic ions is twofold: enzymatically, where

the metals appear to be chiefly associated with enzymes either as activators, such as Mg^{2+}, or tightly bound as catalytic centres in metalloenzymes, such as Cu^{2+}, Zn^{2+} or Fe^{2+}; or structurally, where inorganic species have a role in neutralising electrostatic forces arising from the presence of cellular anionic groups (Jones & Greenfield, 1984). An example of this is the action of Mg^{2+} and Ca^{2+} which shield membrane phospholipids and nucleic acids. Any lack or imbalance of trace metal nutrition may be reflected in sub-optimal growth characteristics or changes in metabolic patterns.

A feature of heavy metal physiology is that even though many metals are essential for growth, they are also reported to have comprehensive toxic effects on cells, mainly due to their ability to denature protein molecules. Metals such as Cu^{2+} and Hg^{2+}, being more electronegative, have relatively high affinities for sulphydryl, imino and amino groups which often form the reactive sites of enzymes (Sadler & Trudinger, 1967) and changes in membrane permeability have been reported (Passow & Rothstein, 1960). In general however, the processes of toxicity are not well understood as the observed responses to excess metal ions in the environment often appear to arise from a complex series of cellular interactions affecting growth, metabolism and viability (Jones & Greenfield, 1984).

When considering metal accumulation it is necessary to know whether transport of a metal ion occurs across the plasma membrane. Clearly, essential divalent metals must cross this cellular barrier in order to be utilised by the microorganism and it is also of interest to know whether non-essential divalent cations can also be taken up into the cytoplasm of cells. Ion uptake in cells is generally thought to be facilitated by metabolism-dependent molecular transport mechanisms

utilising cellular energy and involving translocation of the ion in question from the outer surface of the membrane to the cytosolic surface. Thus, it is necessary to know whether there exists a general transport mechanism for all divalent cations and whether non-essential metal ions can also be transported by such a mechanism (Ross, 1975).

Rothstein (1958) has described the distribution pattern of ions between the cells and the environment as being dependent upon the following factors: (i) binding to anionic groups at the cell surface; (ii) the permeability of the membrane; (iii) the presence of energy-dependent transport mechanisms; (iv) the Donnan potential due to non-diffusible anions in the cytoplasm; (v) reversible ion binding and (vi) binding of ions in a non-exchangeable form. In addition, the movement of specific ions into the cell will be linked to the outward movement of other ions in order to maintain cellular electroneutrality. In an excellent review of yeast ion uptake, Borst-Pauwels (1981) has considered these and other factors in some detail and summarised their effects on membrane transport of mono- and divalent cations.

Despite recent advances in our understanding of some of the theoretical aspects of ion uptake, in practice, information on transport and physiological effects of metals on yeast and fungal cells is still fragmented. The majority of cation uptake studies in yeasts have concentrated on alkali metal cations. However, as early as 1949, the ability of the yeast, *Saccharomyces cerevisiae*, to accumulate Co^{2+} to a concentration approximately 670-fold of that present in the medium was described (Nickerson & Zerahn, 1949). Other early reports from Rothstein's laboratory (Rothstein, 1958,

Rothstein *et al*, 1958) have described the uptake of divalent cations in *S.cerevisiae*. The studies focussed mainly on the transport of Mg^{2+} and Mn^{2+} ions and were designed to determine the involvement of metabolism in transport and the effect of certain inhibitors. Data from a number of subsequent studies indicated that metal accumulation in *S.cerevisia* generally comprised two phases: a rapid and reversible non-specific binding to negatively-charged species present at the cell surface, and a slower, metabolism-dependent intracellular uptake of cations (Fuhrmann & Rothstein, 1968, Ponta & Broda, 1970, Norris & Kelly, 1977). These studies demonstrated a transporter of broad specificity for which Mg^{2+} was a primary substrate whilst other divalent cations, exhibiting lower affinities, were also translocated. The affinities of the metals were only roughly determined.

Our current knowledge of bacterial metal uptake is much clearer. A series of micronutrient transport systems which were highly specific for their respective solutes have been described in a review by Silver (1978). Indeed, Silver (1978) has proposed a unifying hypothesis for ion transport in all microbial cell types. This is that each and every essential metal will have a separate and specific membrane transport system for its cellular uptake. More recently, a specific Zn^{2+} transport system was detected in the yeast, *Candida utilis* (Failla *et al*, 1976). Excepting Failla's study, the majority of yeast metal uptake experiments were carried out using high metal concentrations (around 300 μM or greater), it is clear that under such conditions any specific high-affinity micronutrient systems would be saturated and thus remain undetected. Silver and Jasper (1977) have reinterpreted Rothstein's results with the conclusion that he was observing the Mg^{2+} uptake system analogous to that seen

in bacteria, where Co^{2+}, Mn^{2+} and Zn^{2+} are alternative low-affinity substrates at high concentrations. Consequently, at sub-micromolar levels of essential metals, these specific micronutrient transport systems would be revealed, although, apart from the study of Zn^{2+} uptake in *C.utilis*, there was no documented evidence of this available in yeasts or fungi prior to this study.

There are several other drawbacks to the data obtained on yeast divalent cation uptake. The majority of information is based on work carried out using *S.cerevisiae*. In *C.utilis* there is far less data available, the one report of Zn^{2+} uptake providing contradictory evidence to that obtained using *S.cerevisiae*. A study of Cu^{2+} uptake in *C.utilis* (Khovrychev, 1973) involved relatively high concentrations of Cu^{2+}, around 1 mM. No information regarding cell viability or toxic effects of Cu^{2+} was given and little useful interpretation of these results is possible. Caution should also be exercised when comparing data from the two yeast species. *S.cerevisiae* is a glucose-sensitive yeast (i.e. aerobic respiration is inhibited by high glucose concentrations coupled with the onset of fermentative respiration) whereas *C.utilis* is a respiratory yeast with the major factor controlling respiration being the available oxygen concentration. Clearly, the two species differ somewhat in their metabolic patterns. Furthermore, a number of the early reports of uptake in *S.cerevisiae* utilised commercial samples of baker's yeast. These samples would rarely be pure, with bacterial contaminants often present and would be dubious with respect to the physiological state of the cells.

Some researchers have used metal-resistant mutants, defective in transport properties, for metal uptake studies, although these

present the additional drawback of being atypical organisms. It is well recognised that microorganisms possess the capacity to adapt to increasing concentrations of metals, however genetically stable strains of metal-resistant mutants have also been described (Ross & Walsh, 1981). A wide variety of mechanisms of metal resistance have been documented. Production of melanin in chlamydospores of *Aureobasidium pullulans* has been shown to bind Cu^{2+} ions and have also been implicated in Cd^{2+} resistance (Mowll & Gadd, 1984). Decreased Cu^{2+} uptake has been proposed as a mechanism of resistance in a Cu^{2+} tolerant strain of *S.cerevisiae* (Gadd *et al*, 1984a) and production of hydrogen sulphide resulting in precipitation of insoluble metal sulphide salts has also been reported (Wood, 1984b). Two separate plasmid-borne genes have been shown to be responsible for Cd^{2+} and Zn^{2+} resistance in the bacterium, *Staphylococcus aureus*. One gene codes for a chemiosmotic Cd^{2+} efflux system whilst the second is responsible for the synthesis of a Cd^{2+}-binding protein, similar to metallothionein (Wood, 1984b). There is no evidence for plasmid-mediated metal resistance in yeasts or fungi, although the presence of electron-dense particles within the Cu^{2+}-resistant fungus, *Penicillium ochro- chloron*, indicated intracellular precipitation of Cu^{2+} as a tolerance mechanism (Fukami *et al*, 1983).

A number of factors influenced the choice of organism for this study and the metals used. Whilst there exists a wealth of information on prokaryotic metal transport (Silver, 1978) there is much less data for eukaryotic systems. Yeasts provide useful models as they are readily available, simple to culture, they grow rapidly and much is already known of their genetics, biochemistry and physiology. *C.utilis*, an asporogenous yeast of the family *Crytococcaceae* was the organism used in this study. This yeast is also known at *Torulopsis*

utilis or *Torula utilis* and has been recently reclassified as *Hansenula jadinii*, of which it is the asexual state (Barnett *et al*, 1983). This yeast, which has been grown in continuous culture in the laboratory to produce high-quality edible single cell protein, has several advantages over other yeast species. It is not fastidious in its growth requirements and can utilise carbon from a variety of different sources. It grows well in a synthetic aerobic mineral salts medium without the addition of complex organic substances such as lipids or vitamins. Its aerobic metabolism and active pentose-phosphate pathway predisposes this yeast to a carbon balance in favour of high biomass yields. In addition, as previously detailed, this species has already been subject to some initial studies of metal uptake.

The majority of experiments within this study involved the use of two period 4 transition metals, Mn^{2+} and Cu^{2+}. It is only very recently that the cellular roles and essentiality of these two elements have become clearer and there is still much to be learned. Jones and Greenfield (1984) have suggested that the reason for this poor understanding lies in the two-tiered effects of these metals on structural and enzymatic functions coupled with the occurrence of complex synergistic and antagonistic reactions at both these levels. The requirement for Mn^{2+} in almost all cell types studied so far has been illustrated in a review by Archibald (1986). More specifically, the enzymes: isocitrate dehydrogenase, orotate phosphororibosyl transferase, phosphoprotein phosphatase and pyruvate decarboxylase have all been isolated in yeasts and found to be Mn^{2+}-requiring. 2 to 4 uM Mn^{2+} is required in growth media for optimal growth of *S.cerevisiae*, whilst below 0.02 μM Mn^{2+}, growth is prevented and the fermentation rate rapidly decreases (Jones & Greenfield, 1984).

Omission of Mn^{2+} from the nutrient medium of the fungus, *Aspergillus niger*, results in abnormal hyphal morphology (Kisser *et al*, 1980). Similarly, Cu^{2+} also appears to be an essential metal ion. Shatzman and Kosman (1978) demonstrated a relationship between the cell Cu^{2+} status of the fungus, *Dactylium dendroides*, and the activities of three Cu^{2+}-containing enzymes, galactose oxidase (an extracellular enzyme) and the cytosolic Cu^{2+}/Zn^{2} superoxide dismutase and cytochrome oxidase. The requirement for Cu^{2+} in *C.utilis* has been conclusively shown by Light (1972) and Downie and Garland (1973). In these studies, by chemically-extracting trace Cu^{2+} impurities from the growth medium, Cu^{2+}-limited growth was achieved in continuous culture. Thus, the essential nature of Mn^{2+} and Cu^{2+} makes their study of entry into cells one of particular interest.

It was attempted, at the onset, to avoid being drawn into a largely comparative study, screening a number of yeasts for their relative ability to accumulate a wide range of metals. Instead, focussing interest on an appreciation of the fundamental basis, specificities and kinetic parameters of any transport mechanisms observed, it was decided to concentrate largely on the uptake of Mn^{2+} and Cu^{2+} in the yeast, *C.utilis*. In this manner, an in-depth understanding could be made of the transport of these essential divalent cations under widely varying environmental conditions and during conditions of metal stress and excess. Existing data on metal uptake in yeasts, which is often contradictory could then be evaluated in the light of this study and the relative importance of the non-specific divalent cation transport system in trace metal uptake assessed.

In developing an experimental protocol for monitoring metal uptake it was first necessary to ascertain the toxic action of metals and the

degree of non-specific surface binding as distinct from metabolism dependent transport. This has been covered in Chapter 4 and 5, preceded by an outline of the practical procedures adopted. This is followed by a comprehensive analysis of the specificities and affinities of the transport systems observed and an investigation into the effects of factors such as pH, external ions and the balance between cation influx and efflux. Finally, consideration is given to the regulation of metal uptake systems and its implications for cellular metal homeostasis.

To conclude, it is important to keep in sight the physiological advantages that micronutrient uptake systems would confer on an organism in a natural ecosystem. This situation is far removed from microbial growth in the laboratory and it should be appreciated that "nutrient insufficiency is the most common environmental extreme to which microorganisms are commonly exposed" (Tempest & Neijssel, 1981). Thus, to summarise the objectives of the study, the work was undertaken with the purpose of: (i) systematically investigating divalent cation transport in *C.utilis* and those factors which influence uptake. This included defining the kinetic parameters of transport; (ii) examining for the existence of a general divalent cation transporter in *C.utilis*, as in *S.cerevisiae*, which facilitates trace metal uptake; (iii) investigating whether highly specific metal uptake mechanisms operate for the purpose of scavenging essential trace metals, as seen in bacterial systems; (iv) obtaining information about the mode of regulation of these systems and their role in cellular metal homeostasis.

2. GROWTH OF *CANDIDA UTILIS* IN BATCH CULTURE

2.1. Introduction

Chemoorganotrophs, such as yeasts, have a chemical requirement for growth and synthesis of cellular constituents and an energy-generating system dependent upon oxidation of organic compounds. Unlike glucose-sensitive yeasts, such as *Saccharomyces spp.*; *Candida spp.*, with an extensive oxygen supply, show no glucose repression of respiration and have high growth kinetics and high yields (Fiechter, 1975). *C.utilis* is not fastidious in its nutritional requirements and is capable of oxidising a wide range of carbon catabolites. A carbon and nitrogen source is required together with macronutrients (or major elements) such as phosphorus, sulphur, magnesium, calcium, potassium and sodium, and micronutrients (or trace-elements) such as manganese, copper, cobalt and zinc (Soumalainen & Oura, 1971). The more complex requirements can be satisfied by the addition of yeast autolysate to the medium.

Growth of yeasts in batch culture proceeds through a series of arbitrarily defined phases involving: a) lag phase, when inoculated cells equilibrate to their new environment during which only limited growth occurs; b) exponential phase, when growth is uniform and balanced; c) deceleration phase, when the specific growth is reduced and nutrients in the medium become depleted; d) stationary phase, when nutrients are exhausted, toxic metabolites accumulate, cells begin to lose viability and the growth is so low it only just equals the rate at which cells die.

Most biochemical and physiological studies of yeasts require monitoring the growth of yeast cultures. The absolute measure of growth is dry weight increase, however, by relating increase in optical density to dry weight increase, a close estimation of growth can be made. Growth curves obtained using such a calibration parallel those obtained by direct measurement of dry weight although minor deviations may occur as the physiological status of the cells changes later in the growth curve (Pringle & Mor, 1975). In this section, the batch growth of *C.utilis* is described and the growth parameters determined by an indirect method of estimating dry weight.

2.2. Culturing techniques

2.2.1. Organism

The organism used throughout this study was a strain of the yeast, *Candida utilis*, obtained from the National Collection of Yeast Cultures, Norwich, U.K., catalogue no. NCYC 708.

2.2.2. Media and growth conditions

The solid media used in this study were malt extract agar (MEA) (Oxoid) and MYGP agar of composition (g l^{-1}): malt extract (Oxoid), 3; yeast extract (Oxoid), 3; glucose, 10; mycological peptone (Oxoid), 5; agar (Oxoid, No. 3), 12. Plates and slope cultures were incubated at 30 ^{o}C for 2 days. Unless otherwise stated, the liquid growth medium was of composition (g l^{-1}): glucose, 20; citric acid, 2.8; K_2HPO_4, 5.5; $MgSO_4.7H_2O$, 1; $CaCl_2.6H_2O$, 0.25; $(NH_4)_2SO_4$, 2; $FeSO_4.7H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.00175; $MnSO_4.4H_2O$, 0.0001; $CuSO_4.5H_2O$, 0.0001; yeast extract (Oxoid), 0.1. The pH of the medium was adjusted to 5.5 using saturated KOH. Liquid cultures were grown

at 30 ^{0}C on an orbital shaker at 200 cycles min^{-1}.

2.2.3. Inoculum preparation

Cultures were maintained at 4 ^{0}C on MYGP slopes and subcultured onto fresh slopes each month. 100 ml starter culture in a 250 ml Erlenmeyer flask was grown for 24 h. The culture was then aseptically diluted with sterile distilled water until the resultant cell suspension produced a reading of 0.2 absorbance units (a.u.) (1 cm light-path) at 660 nm on a spectrophotometer (Pye Unicam SP6-400). 400 ml medium in a 1 l shake-flask was inoculated with 0.1 ml of this standard cell suspension and cells were grown overnight until required for harvesting.

As yeasts are prone to cell clumping, prior to all optical density measurements, cells suspensions were exposed to ultrasound in order to disperse the clumps (Pringle & Mor, 1975). By subjecting cells to various periods of sonication and coupled with microscopic assessment of the success of the sonication procedure, an optimal sonication time of 10 sec at a power setting of 3 using a Soniprobe 1130A ultrasonic generator (Dawe Instruments, London, U.K.) was achieved.

2.3. Determination of growth parameters

2.3.1. Optical density / dry weight curve

Triplicate 400 ml cultures were grown overnight until the optical density had attained a value of 0.55 a.u. 100 ml samples were vacuum filtered through dried and pre-weighed cellulose nitrate membrane filters (pore size 0.45 μM, 47 mm diameter, Whatman) and dried at 105 ^{0}C for 16 h prior to reweighing. Thus the dry weight of cells on

a per ml basis of culture medium could be calculated, which correlated to an optical density of 0.55 a.u. A series of dilutions to 10^{-1} was made from further samples of culture suspension and the optical density measured. If the calculated dry weight for a particular dilution is plotted against the optical density of that dilution, an optical density / dry weight curve can be generated (Fig. 2.3.1.).

At higher cell concentrations having optical densities greater than 0.6 a.u., the absorbance fails to increase in proportion to increments in dry weight. Consequently, cell suspensions which had an optical density greater than 0.55 a.u. were diluted to give an optical density value which could be correlated to dry weight directly from the obtained curve.

2.3.2. Growth curves

400 ml cultures in 1 l Erlenmeyer flasks were inoculated and incubated overnight at 30 ^{o}C. Following 10 h incubation growth was barely discernable and a 3 ml sample was removed in order to measure the optical density. Subsequent growth was monitored by regular sampling of the culture medium until after 24 h growth no further increase in optical density could be detected. These optical density values were converted to dry weight measurements by correlation to Fig. 2.3.1. By plotting $\log_{10}$ dry weight as a function of growth time (Fig. 2.3.2.), a growth curve can be constructed which is characteristic of the organism and growth conditions.

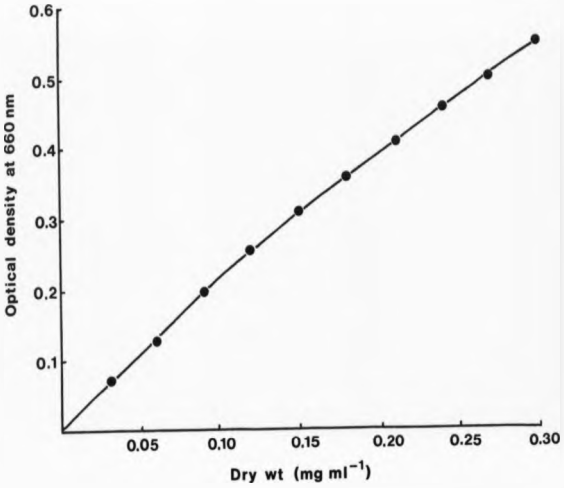


Figure 2.3.1. Optical density / dry weight curve

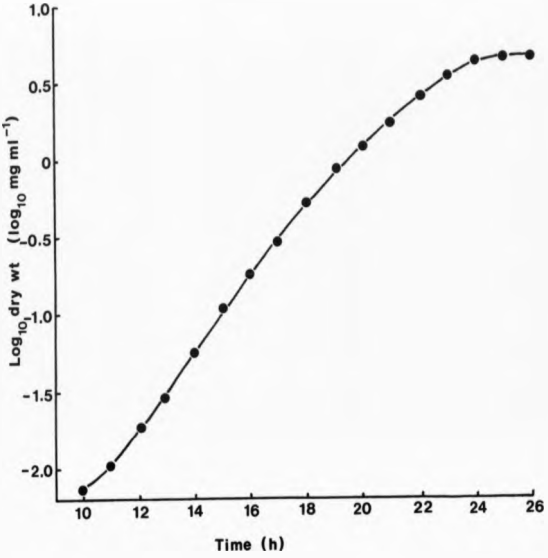


Figure 2.3.2. Growth time / dry weight curve

2.4. pH changes during growth

The pH of the medium at time of inoculation was 5.5. Following 17 h growth, at approximately mid-exponential phase of growth, the pH was slightly reduced to 5.3. However, as the culture age increased further, the pH decreased rapidly until a final value of 3.2 was attained during early stationary phase, following 26 h growth.

2.5. Discussion

C.utilis proved to be a convenient organism for use in this study as its relatively rapid rate of growth, coupled with careful aseptic manipulations, was sufficient to prevent problems of contamination. However, regular microscopic examination of cultures and assessments of culture characteristics such as odour and colour were also carried out in order to reduce the risk of contamination by other yeast species (Pringle & Mor, 1975).

The growth curve generated is typical of growth of cells in batch culture, having a lag phase, an exponential phase and a stationary phase of growth. Numerous repeat cultures demonstrated a high degree of reproducibility of the growth curve, hence the usefulness of the curve in predicting factors such as the state of the culture age and dry weight cells ml^{-1} can be appreciated.

Pringle and Mor (1975) have listed a number of reservations when converting absorbance data to biomass data including the following factors: absorbance is proportional to cell concentration only at low cell densities; a single cell suspension gives different absorbance

values in different spectrophotometers; the dry weight value corresponding to a particular absorbance value is not fixed, but is a function of various properties (ploidy, clumpiness, physiological state, etc.) of the cells. Consequently, whenever accurate determinations of dry weight have been required in this study, direct measurements by filtration of known volumes of cell suspension have been used in preference to estimation using the optical density / dry weight curve.

3. METAL UPTAKE AND QUANTITATIVE ION ANALYSIS

3.1. Introduction

When undertaking any experiment involving the use of low concentrations of metal ions, there are a number of practical aspects to be considered. Contamination by metal ions adsorbed on to surfaces of experimental glassware and in solutions must be avoided and the incubation medium constituents must be carefully chosen to minimise complexation of the metal. In addition, the experimental pH must be sufficiently low to prevent formation of insoluble precipitates of metal hydroxides.

Implicit in the design of cellular uptake experiments is the requirement for several criteria to be met. The incubation conditions should be such that, on addition of the solute under investigation, mixing is quick and efficient to give an even dispersal throughout the cell suspension. Procedures used to sample cells and terminate uptake should be as rapid as practically possible, and finally, ease of operation, particularly during experiments of short duration and rapid sampling time, is important. Whichever experimental design is selected, there is a need for consistency of incubation conditions, particularly when kinetic measurements of transport are required, and throughout this study the protocol outlined in this chapter has been carefully adhered to.

A number of methods are available for the rapid separation of cells from the incubation medium (Kotyk & Janacek, 1975). The technique most frequently employed in microbiological uptake studies is vacuum filtration through cellulose nitrate filters, followed by washing the

cells on the filters with water or buffer in order to remove the incubation solution. Microcentrifugation has also been used (Kotyk & Janacek, 1975, Mowll & Gadd, 1984). In this method, an aqueous suspension of cells is layered on top of a silicone oil or other high-density medium. The density of the oil can be varied such that during centrifugation the cells pass through the oil layer and pellet in the bottom of the centrifuge tube, leaving the aqueous supernatant layered on top. Thus, the technique serves to terminate uptake and wash the cells in one step. Finally, chemical termination, though less commonly used, is worthy of mention. Known transport inhibitors are added to the incubation medium to block uptake. An example of this is the use of uranyl ions to block transport of some monosaccharides in baker's yeast (Kotyk & Janacek, 1975).

A number of analytical techniques have been used in this study to quantify the metal present, according to sensitivity and ease of operation. Atomic absorption spectroscopy (AAS) is the fastest and most convenient method for most routine metal determinations and consequently is the main method used.

Where more sensitive Cu^{2+} determinations have been required, anodic stripping voltammetry (ASV) has been employed. Experiments requiring detection of nanomolar concentrations of Mn^{2+} have necessitated the use of the radionuclide, ^{54}Mn, as the detection of radionuclides is probably amongst the most sensitive of analytical methods available.

3.2. Preliminary procedures

3.2.1. Glassware preparation

Borosilicate glass can act as an ion exchanger, removing metal ions from test solutions *via* wall adsorption (Princeton Applied Research, Technical Note 109A). In order to minimise this source of contamination, all glassware used in sampling and analytical procedures was soaked for 24 h in 4% reagent grade HCl and rinsed in either deionised or distilled water.

3.2.2. Preparation of cell suspension

In general, cells were harvested during mid-exponential phase after approximately 17 h growth when the culture had reached a cell density of 0.3 mg ml^{-1} as determined from the previously described $O.D._{660}$ / dry weight curve (see Fig. 2.3.1.). Cells were collected by centrifugation (4,000 g for 5 min) and washed twice in buffer containing 50 mM glucose and 50 mM 2(N-morpholino)ethanesulphonic acid (MES), adjusted to pH 5.5 with saturated KOH, at 30 ^{o}C. MES buffer was selected as it has a negligible metal-complexing capacity (Good *et al*, 1966) and has been previously used in metal uptake experiments (Baldry & Dean, 1980b, Borbolla & Pena, 1980). Cells were then resuspended in MES buffer and allowed to equilibrate for 30 min prior to experimentation at 30 ^{o}C on an orbital incubator (200 cycles min^{-1}). The cell density was adjusted, as stated for each experiment, such that only a small proportion of the total added metal was taken up during an assay. This proportion varied between assays depending on uptake conditions, however was generally less than 5% and in almost all cases below 10% of the total metal added at the start of the incubation.

3.3. Metal uptake experiments

3.3.1. Experimental procedures

Unless otherwise stated, all uptake assays were carried out in MES buffer and 50 mM glucose at 30 ^{0}C. Uptake experiments were carried out in shake-flasks on an orbital shaker (200 cycles min^{-1}) except those involving the addition of the radionuclide ^{54}Mn, when cell suspensions were incubated in 100 ml flasks on a shaking water bath. All additions to the uptake medium were made to give a final volume of 400 ml in 1 l shake-flasks for most experimental conditions, or 20 ml in the case of radioactive uptake experiments. Following a 30 min equilibration period, a small volume of a metal chloride solution was added. For competition experiments, solutions of metal chlorides were added 1 min before the addition of the metal to be measured. Inhibitors were added 15 min prior to the addition of metals. At time intervals, samples of cell suspension, usually 30 ml were rapidly removed and filtered through pre-weighed membrane filters (47 mm, pore size 0.45 μM, Whatman, U.K.) and washed as described in section 3.3.2. Filters were then dried at 104 ^{0}C for 16 h and reweighed to determine the dry weight of cells in the sample. For experiments involving ^{54}Mn, 1 ml samples were removed, filtered through 0.45 μm filters (15 mm, Whatman, U.K.) and washed as described in section 3.3.2. The filters were then placed in Luckhams tubes prior to counting of radioactivity.

3.3.2. Cell washing

Once cells have been separated from the incubation medium, washing of cells on the filter is important to remove ions adsorbed in the cell wall. A number of washing procedures have been applied in yeast and bacterial uptake experiments: washes with distilled wator (Paton &

Budd, 1972, Fencl *et al*, 1974) or buffer solution (Lusk & Kennedy, 1969, Norris & Kelly, 1977) have been used, as has the use of a washing solution containing EDTA to complex metal ions (Nieuwenhuis *et al*, 1981). An effective technique to remove externally adsorbed ions is by exchanging them with a metal cation not under investigation such as the use of a concentrated wash solution of $MgCl_2$ for Ca^{2+} uptake (Boutry *et al*, 1976) or, in the case of radioisotopic uptake, washing cells with the non-radioactive form of the metal (Fuhrmann & Rothstein, 1968, Nelson & Kennedy, 1971, Failla *et al*, 1976, Borbolla & Pena, 1980).

The effect of washing cells, preincubated for 10 min in MES bufrer amended with 10 μM Cu^{2+} (Ross, 1977), with either 50 ml distilled water or 50 ml of 2 mM $CaCl_2$ solution was investigated. The total Cu^{2+} content of H_2O-washed cells and $CaCl_2$-washed cells was 2.99 ± 0.08 μmol(g dry wt)$^{-1}$ and 2.12 ± 0.06 μmol(g dry wt)$^{-1}$ respectively (values are the mean ± S.E. of 3 separate experiments). The difference between these two values [0.87 μmol(g dry wt)$^{-1}$] represents the amount of Cu^{2+} bound to the cell surfaces which is displaced by the addition of Ca^{2+} ions. In all subsequent experiments involving the uptake of non-radioactive metals, filtered cells were washed with 2 mM $CaCl_2$.

In ^{54}Mn uptake studies, cells were rapidly washed with 10 ml of a chilled solution of 100 μM non-radioactive $MnCl_2$ in order to remove ^{54}Mn ions adsorbed on to the cell walls.

Neither washing procedure removed all surface-bound metal, although it was significantly reduced. The relative efficacy of both washing solutions will be discussed in Chapter 5. It should be noted that no

detectable metal was retained by the filters alone following either of the washing procedures.

3.3.3. Sample digestion

Prior to metal analysis by either AAS or ASV techniques, cell samples on filters were digested to destroy organic matter. The digestion method used was the H_2SO_4 / H_2O_2 technique as described in Procedure B of the Analytical Methods Committee (1967). Following weighing, the dried membrane filters were placed in separate boiling tubes and 0.5 ml conc. H_2SO_4 (AnalaR) added to each tube. The tubes were placed on a heating block at 350 ^{0}C until the filters and samples charred. 3.0 ml of 60% H_2O_2 were added dropwise after removal from the heating block. The samples were then reheated until the H_2O_2 had boiled off and white fumes appeared from the conc. H_2SO_4. Further additions of H_2O_2 were made if there was any evidence of a residual yellow colouration due to organics present. Digestion was complete when the acid remained colourless after heating. After cooling, the boiling tubes were washed down with distilled water and the contents transferred to 5 ml volumetric flasks and made up to volume with distilled water. This technique enabled the total metal content present in the sample to be determined.

By analysing digests of the filters alone, their basal metal content could be established. The Whatman filters contained no detectable copper or manganese, however, a small quantity of zinc (< 0.1 µg Zn^{2+} g^{-1} filter) was found to be present.

3.3.4. Chemicals

Throughout this study, the chemicals used were of AnalaR or best available grade. Growth media, where stated, were purchased from Oxoid Ltd., Wade Rd., Basingstoke, Hampshire, U.K. and all other chemicals were obtained either from BDH Chemicals Ltd., Poole, U.K. or from the Sigma Chemical Company Ltd., Poole, Dorset, U.K.

Metal chloride salts were used in all uptake experiments. 1 M stock solutions of metal salts were made up in 1 N HCl and stored at 4 ^{o}C. Fresh working solutions of metals were prepared for each experiment and made up either with distilled water or 1 M HCl.

3.3.5. Experimental pH

The experimental pH for all work was 5.5 unless otherwise stated.

3.4. Quantitative ion analysis

3.4.1. Atomic absorption spectroscopy

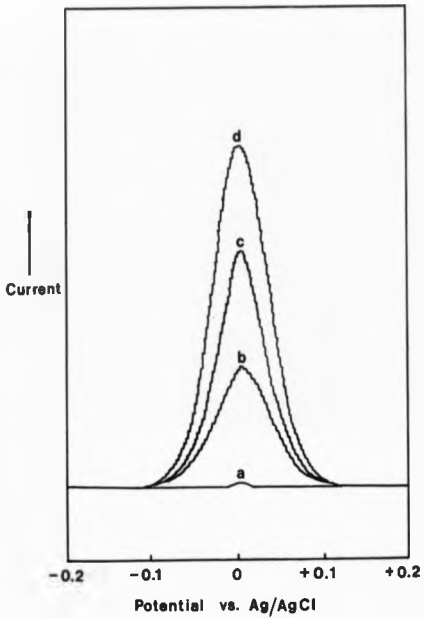
Assays for total metal content were routinely carried out using AAS on a Perkin-Elmer Atomic Absorption Spectrophotometer model 280 with an air-acetylene flame. 10% H_2SO_4 was used as a blanking solution and standard metal solutions were prepared in 10% H_2SO_4 in all cases. Copper, manganese, cobalt and potassium were determined in the range, 0 - 2.0 $\mu g\ ml^{-1}$, and zinc in the range, 0 - 1.0 $\mu g\ ml^{-1}$, since in these ranges the relationship between metal concentration and absorbance is linear.

3.4.2. Anodic stripping voltammetry

Voltammetry is a technique which allows determination of species in a solution that can be electrochemically oxidised or reduced. A potential is applied to the sample *via* a conductive, working electrode and a linear voltage ramp scans the potential over a region of interest. If, at a particular potential, a metal is reduced, a current will flow at the working electrode. The potential at which this occurs is characteristic of the redox potential of the metal ion and the amount of current produced is proportional to the free ion concentration in solution.

Anodic stripping voltammetry has been previously used in a number of studies of cellular metal uptake in microorganisms (Khovrychev, 1973, Aickin & Dean, 1978). The technique involves two steps (Matson & Roe, 1967): a deposition (reduction) step during which the metal ion is concentrated from the bulk solution by electrodeposition on to the electrode, forming an amalgam in a thin mercury layer on the electrode surface; this is followed by a stripping (oxidation) step, when a linear anodic voltage scan is applied, during which the metal is removed and the amount deposited on the electrode is measured from the recorded current curve (Fig. 3.4.2.). A quantitative measurement of the free electroactive metal ion can be determined from the current peak height relative to peak heights produced by standard additions, such that a standard curve can be constructed by plotting peak height against added metal concentration. An advantage of using voltammetric techniques is the linear response to metal ion concentration, often over three or four orders of magnitude. Hence, sample dilution, often necessary when using other detection techniques and creating a further source of error, is avoided.

Figure 3.4.2. A typical anodic stripping voltammogram obtained for Cu^{2+} determinations



a - Cu^{2+}-free buffer

b - 50 nM Cu^{2+}

c - 100 nM Cu^{2+}

d - 150 nM Cu^{2+}

ASV measurements were performed on a Metrohm 663 VA stand with a hanging drop mercury electrode (HMDE) coupled to Metrohm 626 Polarecord analyser. Deoxygenation was carried out with oxygen-free-nitrogen for 5 min. ASV was carried out using a linear sweep on differential pulse mode 50. The scan rate was 10 mV sec^{-1} and the current sensitivity range generally set at 5 nA. Copper determinations were carried in 10% H_2SO_4. The initial potential was set at - 0.50 V and scanning was carried out in the positive direction, the redox potential for copper occurring at 0 V.

3.4.3. Radionuclide measurement

^{54}Mn, purchased from Amersham International Ltd. (Amersham, Bucks., U.K.), was in the form $MnCl_2$, having an initial activity of 509 mCi mg^{-1} Mn^{2+}. ^{54}Mn stock solutions were prepared with distilled water, each having an activity of 11 $\mu Ci\ ml^{-1}$. As ^{54}Mn is a hard gamma emitter having an emission energy of 0.835 MeV, no scintillant fluid was required and radiation measured directly. Radiation counts from samples in Luckhams tubes were measured in a Packard Auto-Gamma Scintillation Spectrometer (Packard Instrument Co., U.S.A.). The counting time was adjusted such that over 5,000 counts were detected and, in general, was for 5 min. The normal background count was automatically subtracted from all experimental counts. The radioioactivity of known concentrations of ^{54}Mn was measured in order to produce a conversion factor to determine Mn^{2+} concentrations from experimental counts.

3.4.4. Trace metal impurities

One problem encountered in experimental work involving nanomolar concentrations of metal ions can be the effect of trace metals, present in media constituents as impurities. In this study, the majority of uptake experiments used a simple MES buffer, generally containing analytical grade glucose and pH-adjusted using ultra-pure Aristar grade KOH. This buffer contained no detectable Cu^{2+} or Mn^{2+} contaminants, even when concentrated 100-fold by rotary evaporation, as ascertained by both AAS and ASV techniques.

In addition, it is worthy of mention here that unavailability of metal to the cells, through adsorption to glass reaction-vessel surfaces or by complexation to buffer constituents, was minimal. Prolonged ^{54}Mn uptake experiments, using a high cell density suspension, recovered almost 100 % of added radioactivity, demonstrating that virtually all the ^{54}Mn was in an available form.

4. ASSESSMENT OF METAL TOXICITY

4.1. Introduction

Essential metals such as Co^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Ni^{2+} are unusual in that they belong to a group of substances capable of exerting a biphasic effect on cells (Albert, 1979). In their absence, or in metal-deficient conditions imposed by the environment, their lack of availability to the cell causes impairment of cellular functions. Similarly, at high concentrations, once the cell's buffering capacity for essential metal ions is exceeded, toxicity becomes evident. In most environments, due to the minute cellular requirements for a number of metals, deficiency of a certain trace element is unlikely to be the major growth-limiting factor, however growth inhibition due to high concentrations of metals is more common. Indeed the oligodynamic properties of heavy metals have long been exploited; Cu^{2+}- and Hg^{2+}-containing fungicides have been in use for a number of years.

Essential metals, in eliciting a biphasic response in cells, are capable of growth stimulation at concentrations greater than those normally present in growth media. Enhanced growth of *Aspergillus niger* and *Aspergillus conoides* on agar plates containing up to 10 mM and 1.8 mM Mn^{2+} respectively has been observed (Babich & Stotsky, 1981, Townsley, 1985). Co^{2+}, Cu^{2+}, Ni^{2+} Zn^{2+} and Mn^{2+} all have stimulatory effects on growth and fermentation in *Saccharomyces spp.*, the optimum concentrations for growth being in the range 0.1 μM to 10 μM (Jones & Greenfield, 1984). It is possible that the stimulation of growth may be due to a requirement for more metal than is available from the normal micronutrient concentrations in the

medium or that the metal induces an increase in the membrane permeability which allows for a freer flow of nutrients into the cell with a concomitant increase in metabolism.

In addition to the essential metals, there exist a number of cations for which the cell has no requirement, such as Cd^{2+}, Hg^{2+}, Pb^{2+} and Os^{2+} (Jones & Greenfield, 1984), and which have no reported stimulatory effects but which act as inhibitors of growth and metabolism at varied concentrations. In general, toxic effects become evident at much lower concentrations for non-essential metals, which in the natural environment seldom reach concentrations in excess of 1 $\mu g\ g^{-1}$ (Wood & Wang, 1983). One explanation for this is that non-essential, toxic ions may physically and chemically resemble essential ions and be recognised as such by the cell, binding to those cellular sites normally occupied by the latter type of cation where they interfere with normal cell processes (Sadler & Trudinger, 1967, Wood & Wang, 1983).

At a molecular level, the toxicity of metals such as copper, mercury and silver has been attributed to binding to sulphydryl, amino and imino groups, often present at the catalytic sites of enzymes (Ross, 1975). Sulphydryl groups are essential for the maintenance of the tertiary and quaternary structure of many proteins, consequently binding of metals may disrupt the configuration of metabolically important protein moieties. Heavy metals produce a wide range of biochemical and morphological effects on cells when present at sub-lethal concentrations. Copper ions can convert bacterial rods into spherical forms (Sadler & Trudinger, 1967) and the effects of continuously culturing *C.utilis* in 1.2 - 1.6 mM Cu^{2+} include; greatly increased cell size, cell wall thickening, the disappearance of

mitochondrial cristae and the inclusion of dense copper-containing particles in the vacuole and cytoplasm (Khovrychev *et al*, 1977). Inhibition of respiration and fermentation activities in *S.cerevisiae* was caused by Cd^{2+} and Hg^{2+} concentrations in excess of 1 μM and 50 μM respectively (Grafl & Schwantes, 1983a) whilst Gadd *et al* (1984a) reported a decrease in respiration rate in cells of *S.cerevisiae* following 2 h incubation in 5 μM $CuSO_4$. Inactivation of yeast enzymes by metal ions has been widely reported, in particular, Cd^{2+} and Zn^{2+} have been shown to inhibit malate dehydrogenase and glutamate dehydrogenase (Grafl & Schwantes, 1983b). Cd^{2+}, Zn^{2+} and Cu^{2+} inhibit synthesis of RNA, ribosomes and cell protein (Khovrychev *et al*, 1974, Berthe-Corti *et al*, 1984) and Cu^{2+} reduces the amount of cytochrome a, b and c in yeast cells (Khovrychev *et al*, 1974). Khovrychev and Rabotnova (1972) concluded that Cu^{2+} ions inhibit one 'bottleneck' enzyme reaction in *C.utilis* on the basis that growth inhibition by Cu^{2+} obeys the equation of non-competitive inhibition of enzyme reactions. However it seems unlikely that the action of Cu^{2+} is so precise; indeed, Cu^{2+}, Cd^{2+}, Zn^{2+} and Ag^{+} ions all cause membrane disruption, as detected by the loss of K^{+}, Mg^{2+} and u.v.-absorbing substances, such as nucleotides, from yeast cells (Golubovich *et al*, 1976, Macara, 1978, Gadd & Mowll, 1983, Mowll & Gadd, 1983), whilst Mn^{2+}, Ni^{2+} and Co^{2+} appear to have less effect (Joho *et al*, 1984). There is little information available on the toxicity of manganese to yeasts and fungi although one study details the inhibition of protein synthesis and nuclear DNA replication in *S.cerevisiae* by the action of 10 mM Mn^{2+}. In addition, a number of mutagenic effects have been described (Putrament *et al*, 1977).

The effect of metal ions is often primarily observed by their effect on growth, either on agar plates or in liquid culture. In liquid

culture, effects are usually manifested by an extended lag phase prior to the onset of growth (Ross & Walsh, 1981), in a decrease in growth rate or mean generation time or in a reduced growth yield (Jones & Greenfield, 1984). Table 4.1. summarises the range of metal ion concentrations which become inhibitory to the growth of yeasts and fungi in liquid culture. The response to metals varies widely over a range of concentrations although the data does give an indication of the relative magnitude of the effects of various cationic species. Mn^{2+} and Zn^{2+} seem to be fairly innocuous in their toxicity to cells whereas Cu^{2+} and Cd^{2+} exert their effects at much lower concentrations. Considerable caution should be exercised when comparing the toxic effects of various metals, as they are greatly influenced by a number of factors including pH, the ability to internalise a metal and the presence of other related ionic species or complexing agents. Thus, it is impossible to predict with any accuracy the concentration at which a given metal ion will become toxic (Ross, 1975).

It is clear that penetration of the cell by a metal ion, whether by transport across the cell membrane or simply passive diffusion, is likely to enhance its toxic action. An indication of this is the dramatic decrease in viability of *S.cerevisiae* observed when glucose is added to a cell suspension containing 16 μM Cu^{2+} (Ross, 1977). Hence an intimate relationship exists between toxicity and cellular uptake of many metals although there are exceptions; the inhibition of yeast metabolism by uranyl ions is due to reduced hexose uptake, little or no uranium is taken up by the cell (Jennings, 1963). Whilst it is important to appreciate this relationship, both toxicity and uptake will be discussed separately, as a major part of this study is devoted to the cellular uptake of metal species.

Table 4.1. The effect of divalent cations on the growth in liquid culture of a number of yeasts and fungi

Organism	Cation	Minimal concentration at which inhibition observed (mM)	Reference
Candida sp.	Ni^{2+}	0.085 - 0.17	Babich & Stotzky 1983
Saccharomyces sp.		>0.1	Jones & Greenfield 1984
Saccharomyces sp.	Mn^{2+}	>10.0	Jones & Greenfield 1984
Aspergillus sp.	Zn^{2+}	0.1 - 1.0	Townsley 1985
Penicillium sp.		1.0	Townsley 1985
Trichoderma sp.		1.0	Townsley 1985
Candida sp.		3.8 - 4.6	Andreeva *et al* 1980
Saccharomyces sp.	Cu^{2+}	>0.01	Jones & Greenfield 1984
Penicillium sp.		0.01 - 0.1	Townsley 1985
Saccharomyces sp.		0.03	Ross & Walsh 1981
Candida sp.		0.05	Ross (unpublished data)
Saccharomyces sp.		0.08 - 0.31	Sahinkaya 1960
Aspergillus sp.		0.1 - 1.0	Townsley 1985
Candida sp.		0.16 - 0.39	Babich & Stotzky 1983
Candida sp.		0.3 - 0.4	Khovrychev 1972
Saccharomyces sp.	Co^{2+}	0.09	Jones & Greenfield 1984
Saccharomyces sp.		>0.1	Heldwein *et al* 1977
Rhodotorula sp.		1.0	Joho 1975
Penicillium sp.	Cd^{2+}	0.001 - 0.01	Townsley 1985
Saccharomyces sp.		0.009	Berthe-Corti 1984
Aspergillus sp.		0.01	Townsley 1985
Rhodotorula sp.		0.09	Berthe-Corti 1984
Trichoderma sp.		0.1 - 1.0	Townsley 1985

pH plays a critical role in metal toxicity. At high pH values, formation of metal hydroxide and oxide precipitates reduce metal availability to the cell. At acidic pH, free metal ions exist in solution although increased competition for metal binding sites by H^+ ions, coupled with a decrease in the divalent cation concentration near the cell membrane due to the decreased negative surface potential (Borst-Pauwels, 1981) may lead to decreased binding and uptake. Consequently, there appears to be a critical pH range for toxicity. Starkey (1973) demonstrated maximum growth inhibition by copper at pH 4.2 to pH 5.0 and suggested that this range reflects maximal copper uptake under these conditions. Amelioration of copper toxicity in conjunction with decreasing pH has also been observed in *Penicillium ochro-chloron* (Gadd & White, 1980) whilst reduction of the initial pH from 4.5 to 3.2 greatly enhanced growth of *Aureobasidium pullulans* in the presence of 4 mM Cu^{2+} (Gadd & Griffiths, 1980).

The bioavailability of metal species can also be affected by complex formation with medium constituents and when metal-binding compounds such as EDTA, oxine and a number of amino acids are present, toxicity to yeasts and fungi is usually markedly reduced (Avakyan, 1971, Starkey, 1973, Imahara *et al*, 1978) and the significance of this metal chelation in the natural environment has been discussed in a useful review by Sadler and Trudinger (1967). One further factor is the series of interactions between related metal cations which serves to promote synergism or antagonism of toxicity. For example, low Zn^{2+} concentrations reduce Cd^{2+} toxicity in *S.cerevisiae* whilst high Zn^{2+} concentrations intensify the effects of Cd^{2+} (Grafl & Schwantes, 1983c). Cu^{2+} ions have been reported to enhance the toxic effects of both Cd^{2+} and Ni^{2+} ions whereas Mn^{2+} reduces the effects of Cd^{2+} and

Cu^{2+} (Sahinkaya, 1960, Babich & Stotzky, 1983). The mechanism of these interactions is still unclear, although it may be primarily due to an increase or decrease of metal uptake (Webb, 1968, Joho, 1975, Kessels *et al*, 1985) and this aspect will be discussed in a later chapter. Alternatively, it has been postulated that they represent direct competition between essential and toxic ions for enzyme activation sites within the cell (Sadler & Trudinger, 1967).

In conclusion, the importance of understanding metal toxicity should be underlined here; in any study of metal uptake, the toxic effects on the cell must be ascertained and minimised in order that cellular metal transport systems are observed without hindrance. In this section, the effects of several divalent cations on cells of *C.utilis* are investigated. In particular, Cu^{2+} and Mn^{2+}, two metals with widely differing toxic actions, are studied as a preliminary step towards appreciating their mode of internalisation.

4.2. Materials and methods

4.2.1. Assessment of viability

In order to assess the effects of Cu^{2+} and Mn^{2+} on cell viability, a plate count method was used. Cells, harvested at mid-exponential growth phase and washed with distilled water, were resuspended in MES buffer and 50 mM glucose, pH 5.5, to a cell density of 1 mg ml^{-1}. Metal solutions were added to the cell suspension to give a final volume of 100 ml in 250 ml Erlenmeyer flasks. Following 30 min incubation at 30 ^{o}C on an orbital shaker (200 cycles min^{-1}, a 5 ml sample was removed and sonicated in the manner previously described to disperse clumps of cells. It was previously established that the

sonication procedure did not affect cell viability or the susceptibility of cells to metal poisoning. The sample was diluted by serial dilution in sterile distilled water to 10^{-6}. A 1 ml sample from each dilution to 20 ml sterile molten malt extract agar at 45 ^{o}C, mixed in a petri-dish and allowed to set. All pour-plates were incubated at 30 ^{o}C for 2 days and the number of resultant colony forming units counted.

4.2.2. Respirometry

Following 30 min incubation in the presence of a metal or a mixture of metals in MES buffer and 50 mM glucose, pH 5.5, as previously described, 5 ml cell samples were removed and the oxygen uptake rate measured using an oxygen electrode. A Rank oxygen electrode was connected to an MSE Spectroplus oxygen meter with a recorder attachment. To calibrate the instrument, 5 ml MES buffer was added to the reaction vessel and stirred with a magnetically coupled stirrer bar. After several minutes equilibration period, the sensitivity of the Spectroplus was adjusted to give a full scale deflection at 100% saturation and the zero oxygen content of the buffer set by adding sodium dithionite crystals to the reaction vessel. A rapid response time of 15 sec was attained for the instrument to measure a difference in oxygen content from 100% to 0 - 5%. Water, at 30 ^{o}C, was passed through the water-jacket to maintain the temperature of the cell sample. Following each sample addition, the calibration of the instrument was checked and the reaction vessl rinsed with distilled water. The cell density of the suspension was adjusted such that oxygen uptake could be measured over a period of 5 to 10 min. The absolute rate of oxygen uptake was determined from the known oxygen content of air-saturated distilled water [7.63 mg O_2 l^{-1} at 30 ^{o}C (WTW Instruction Manual, 1979)].

4.3. Results

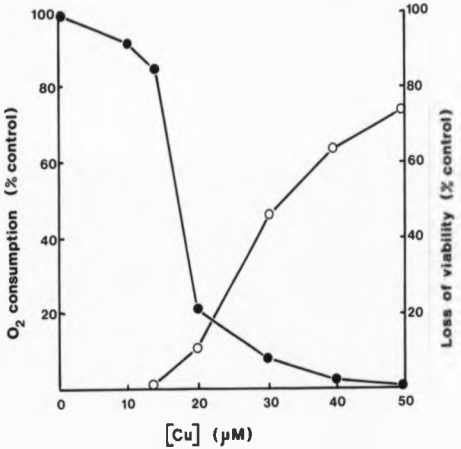
4.3.1. Effect of copper and manganese on cell viability

Figures 4.3.1. and 4.3.2. show the effect of varying the Cu^{2+} or Mn^{2+} concentration on the viability of *C.utilis*. Copper became lethal at 20 μM, where 10% loss of viability was recorded. Cell death increased rapidly up to 50 μM Cu^{2+} when only 26% of the population remained viable. Manganese, on the other hand, did not affect viability until it reached a concentration of 50 mM which reduced viability by 39.2%, 85% loss of viability occurring at 100 mM Mn^{2+}. Co^{2+} exerted a similar effect to Mn^{2+} with 32% and 88% loss of viability at 30 mM and 100 mM respectively.

4.3.2. Effect of divalent cations on oxygen uptake

The inhibition of respiration by Cu^{2+} and Mn^{2+} is shown in Figures 4.3.1. and 4.3.2. In 10 μM Cu^{2+}, there is a slight decrease in respiration rate of 12%, similarly, at 14 μM Cu^{2+}, respiration is inhibited 16%. However between 14 μM and 20 μM Cu^{2+}, oxygen uptake is considerably reduced with only 21.8% of respiration in the absence of Cu^{2+} occurring. At 50 μM Cu^{2+} no oxygen uptake occurred. Inhibition of respiration by Mn^{2+} becomes evident at 50 mM whilst 100 mM Mn^{2+} almost completely inhibits oxygen uptake. Table 4.3.2. summarises the effect of low concentrations of Mn^{2+}, Zn^{2+}, Co^{2+}, Mg^{2+} and Ni^{2+} on respiration. Cu^{2+} was also added to the incubation medium to investigate any synergistic or antagonistic effects of a mixture of metals on oxygen uptake. None of the metals examined were inhibitory at the concentrations studied, with the possible exception of Ni^{2+} where a small reduction in respiratory activity was observed at 100 μM.

Figures 4.3.1. and 4.3.2. The effect of Cu^{2+} (Fig. 4.3.1.) and Mn^{2+} (Fig. 4.3.2.) on respiration (●,■) and viability (○,□). Values represent the means of duplicate experiments.



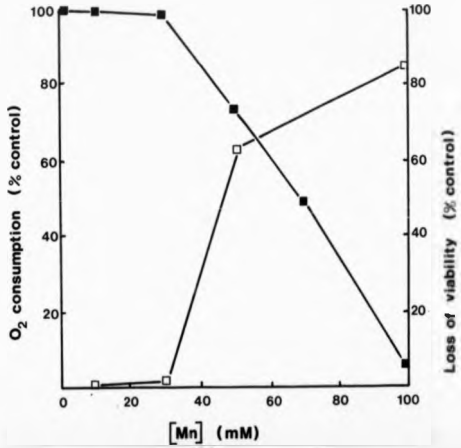


Table 4.3.2. Effect of divalent cations on oxygen uptake

Metal ion (μM)	Oxygen uptake (% control)
Cu^{2+} 5	99.3
Mn^{2+} 50	97.1
Mn^{2+} 100	95.9
Mn^{2+} 50 + Cu^{2+} 5	98.6
Zn^{2+} 50	99.3
Zn^{2+} 100	91.9
Zn^{2+} 50 + Cu^{2+} 5	100.7
Co^{2+} 50	102.2
Co^{2+} 100	99.3
Co^{2+} 50 + Cu^{2+} 5	99.3
Mg^{2+} 50	105.1
Mg^{2+} 100	101.4
Mg^{2+} 50 + Cu^{2+} 5	103.4
Ni^{2+} 50	89.7
Ni^{2+} 100	79.7

Values shown (mean of duplicate experiments) are expressed relative to controls. The control value for the respiration rate in the absence of any metals was 76.3 ± 0.5 nmol O_2 min^{-1} (mg dry wt)$^{-1}$ (mean ± S.E. of 5 determinations).

4.4. Discussion

Yeast cells provide simple eukaryotic models in which to investigate metal toxicity. Indeed, their sensitivity to metals forms the basis of an assay to quantify the toxicity of heavy metals (Bitton *et al*, 1984) and in this study the parameters used to examine toxicity were cell viability and respiration. The main advantage of the methodology used was that it enabled incubation of the cells with metals to be carried out in a non-complexing medium which allowed for quantitative and comparable results. Toxicity tests on solid media have met with some success (Gadd, 1983) although results obtained are usually qualitative (Somashekar *et al*, 1983). In this study, *C.utilis* was highly susceptible to copper poisoning whilst manganese had little effect. These results compare favourably with the general trend of metal toxicity observed in other species (see Table 4.1.). The *C.utilis* strain used was less sensitive to Cu^{2+} than a wild-type strain of *S.cerevisiae* in which oxygen uptake was inhibited 43.5% by 5 μM $CuSO_4$ (Gadd *et al*, 1984a). In the same study, a Cu^{2+}-resistant mutant of *S.cerevisiae* was isolated, however its respiratory rate was inhibited some 40% by 10 μM $CuSO_4$ although viability of the mutant strain in 5 μM and 10 μM $CuSO_4$ was far greater than the wild-type strain. Growth inhibition of *C.utilis* in batch culture by Cu^{2+}, as demonstrated by an increase in generation time, became evident at a concentration of 50 μM (Ross, unpublished observations) and this is in agreement with this study.

It is important to understand the effect of combinations of metals on cells if the action of competing cations on metal uptake is to be subsequently investigated. In this case, no inhibition of oxygen uptake was observed with any metal in combination with copper.

Hence, these metal concentrations could be used in uptake specificity and competition experiments whilst the use of Ni^{2+} ions at concentrations of 50 μM or greater was ruled out by this study.

The difference in toxicity between Cu^{2+} and Mn^{2+} is most probably attributed to their chemical properties. More electronegative elements such as Cu^{2+} and Hg^{2+} have high affinities for active sites on enzymes and are likely to be more toxic (Ross, 1975). The order of electronegativities of divalent metals is:
Hg > Cu > Sn > Pb > Ni > Co > Cd > Fe > Zn > Mn > Mg > Ca > Sr > Ba
(Bowen, 1966) whilst Somers (1959) reported an order of toxicity for *Botrytis fabae* spores of:
Hg > Cu > Pb > Ni > Co > Zn > Mn > Sr > Mg, Ca > Ba.
Clearly there is some correlation between these two series, however instances of exceptions to this rule have been reported (Townsley, 1985) and other factors such as solubility products and chelate stabilities of metals (Bowen, 1966) or species more tolerant to a specific metal (Trevors *et al*, 1985) may well affect the relative toxicity.

It should be remembered that experimental design may affect the detection threshold of inhibitory action of a metal. In this study, quantitation of the toxic effects on cells was preceded by a 30 min period of incubation with the metal. If a longer contact time had been used, it is possible that subsequent respiratory inhibition may have been greater for a given metal concentration. A 30 min incubation was considered a suitable period for the purposes of this study as this was the maximum time over which metal uptake was to be measured. Thus, comparisons with studies using similar techniques but differing in incubation times are often invalid. If toxicity

tests are to be accurately interpreted they must be carried out under conditions identical to those in which uptake studies will be carried out.

It is clear that studies of this nature are an essential prerequisite to metal uptake studies. One study of Cu^{2+} uptake in *C.utilis* (Khovrychev, 1973), used concentrations of Cu^{2+} in excess of 1 mM. Although the cell density used was 3 mg ml^{-1}, this concentration does seem rather high and no details of cell viability were given. Oxygen uptake and viability tests in this present study demonstrate total inhibition of respiration and 74% loss of viability at a Cu^{2+} concentration of 50 μM, hence subsequent experiments were carried out at lower concentrations in order to minimise cell damage. Viability can give a gross indication of the magnitude of toxicity yet inhibition of growth and metabolism can occur at concentrations well below the lethal threshold. For example, with reference to Figure 4.3.1.; if a study of Cu^{2+} uptake was to be carried out at a Cu^{2+} concentration of 20 μM, where 10% loss of viability was to be considered acceptable, then any Cu^{2+} uptake under these conditions might be seriously affected as oxygen uptake is reduced by nearly 80%. Once details were available of non-toxic metal concentrations under the experimental conditions imposed, the next section of this study was an investigation of the cellular uptake and accumulation of these metals.

5. SURFACE BINDING AND ENERGY-DEPENDENT UPTAKE OF MANGANESE AND COPPER

5.1. Introduction

Initially, cell-metal ion interactions occur at the cell's periphery, that is, the cell wall and plasma membrane, in most instances leading to ultimate internalisation of the metal. In this chapter, the surface binding and cellular uptake of metals by yeasts is discussed and the energy-dependence of Mn^{2+} and Cu^{2+} uptake in *C.utilis* demonstrated. In view of the numerous terms that have been applied in studies of interactions of metals with cells, the meaning of several terms used in this text will be defined as suggested by Failla (1977). The term, "accumulation", is applied to the total amount of metal associated with the cells. No distinction is made here whether the metal is surface-bound or internalised. The term, "uptake", is synonymous with influx, ie. the process of internalisation of the metal within the cells.

Binding of cations to anionic groups at the cell surface has been of increasing interest in recent years as a result of the possible application of microbial biomass for the removal of toxic heavy metals or the recovery of valuable metals from aqueous effluents (Brierley & Brierley, 1983). A number of chemical groups act as potential cation binding sites and these include phosphoryl, carboxyl, imidazole, sulphydryl, phenolic hydroxy, and amino groups (Rothstein & Hayes, 1956). The relative affinities of cations for yeast binding sites most probably reflects the relative stability constant for an interaction between a specific metal ion and a particular group. Rothstein and Hayes (1956) have shown the relative

binding affinities of cations for baker's yeast to be UO_2^{2+} >> Ba^{2+} > Zn^{2+}, Co^{2+} > Mg^{2+}, Ca^{2+}, Sr^{2+} > Mn^{2+}, Cu^{2+}. UO_2^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} ions appear to bind to cell wall polyphosphates and carboxyl groups on walls and cell surface proteins (Rothstein & Hayes, 1956, Van Steveninck & Booij, 1964, Paton & Budd, 1972) whilst thorium binds not only to polyphosphates, but also to phosphatides in the cell membrane (Van Steveninck & Booij, 1964). Uranium can also coordinate with the amine nitrogen of the chitin component of the fungal cell wall (Tsezos & Volesky, 1981). In addition, Zn^{2+} and Hg^{2+} would be expected to exhibit special affinities for imidazole and sulphydryl groups respectively (Rothstein & Hayes, 1956).

Using electron spin resonance (ESR) techniques, Tsezos (1983) reported that at least two oxygen atoms and one nitrogen was involved in a chitin-copper coordination complex whilst ESR spectra of Raynor and Townsley (personal communication) indicated that copper ions coordinated to either 4 nitrogens or 2 nitrogens and 2 oxygens in the cell wall of *Penicillium spinulosum*, with no observable sulphur involvement. By selective chemical modification of the cell wall of the bacterium *Bacillus subtilis*, Doyle *et al* (1980) suggested that carboxyl and amino groups were important in regulating interaction between cation and cell walls, whilst the selective removal of phoshate from teichoic acids drastically reduces the ability of bacterial walls to bind divalent cations (Heptinstall *et al*, 1970). The only corresponding study using fungal cell walls proved inconclusive (Townsley, 1985).

pH may significantly affect binding to various groups. At pH 4, primary amines would be positively charged and would not be expected

to interact with cations and many of the carboxylate groups would be neutral. However above pH 3, most of the phosphate groups present as mono- or diesters would have a negative charge and be capable of forming bonds with metal ions (Tobin *et al*, 1984). These primary bonds may be augmented by weaker associations with hydroxyl and other groups.

Several other physical and physiological factors can affect the amount of metal which binds to the cell surface. The cell wall composition can be changed by altering the environmental growth conditions (Ellwood, 1970) and by growing *Bacillus subtilis* and *Pseudomonas fluorescens* in a variety of limiting nutrients, the binding of copper could be increased as the limiting nutrient was changed in the order, carbon < magnesium < nitrogen < potassium (Baldry & Dean, 1981). Clearly, different cell types can bind differing amounts of metals. This is seen in the greatly elevated Cd^{2+} binding which occurs in thick, melanised walls of chlamydospores of *Aureobasidium pullulans*, as compared to the Cd^{2+} binding capacity of yeast-like cells and mycelium (Mowll & Gadd, 1984). In addition, copper binding is reduced by the presence of complexing amino acids, especially histidine and cysteine (Wakatsuki *et al*, 1985), and also by the concentration of yeast cells in aqueous suspension. The latter effect is thought to be due to increased electrostatic interaction between cells at high cell densities (Itoh *et al*, 1975).

Surface binding of metals, which is both rapid and reversible (Rothstein & Hayes, 1956), can be analysed using Scatchard mass/law plots (Scatchard, 1949), by fitting the data to the Langmuir adsorption model (Langmuir, 1918) or by applying Freundlich's adsorption isotherm (Freundlich, 1926). In view of the fact that

Langmuir's model was derived to relate the adsorption of gas molecules to solid surfaces and that Freundlich's isotherm is a purely empirical formula, extrapolation of these methods to analyse adsorption of metals in microbial systems should be viewed with some circumspection. However, using these techniques, two types of binding sites, one of high affinity and one of low affinity for the metal, have been demonstrated for Cu^{2+} in the yeasts, *C.utilis* (Khovrychev, 1973) and *Debaryomyces hansenii* (Wakatsuki *et al*, 1979) and in the bacterium *Escherichia coli* (Baldry & Dean, 1980b), and for Mn^{2+} in baker's yeast (Rothstein & Hayes, 1956). It is possible that the high affinity site represents the site of transport for the metal. Indeed, in metabolising cells of the alga *Chlorella fusca*, the high affinity component of Zn^{2+} accumulation is thought to be due to the energy-dependent entry of Zn^{2+} into cells (Broda, 1972).

Several roles for metal wall binding have been suggested. Specific divalent cations present in cell walls may have essential roles in maintaining the integrity of the surface structure. Metal ions may stabilise the cell wall-inner membrane attachment sites and are required by extracellular enzymes for structural and catalytic purposes (Failla, 1977). Another possible role for metals in the cell wall involves stabilising cell attachment-separation forces between cell surfaces and other objects or cells by either reducing the negative potential or altering the conformation of surface macromolecules (Failla, 1977). Copper tolerance in the atypical fungus *Penicillium ochro-chloron* has been attributed to the massive binding and deposition of copper in the surface system (Fukami *et al*, 1983).

In addition to cell wall binding, the majority of metal ions are internalised. The processes of movement of ions or molecules into cells has been widely studied and the general transport paradigm which will be described encompasses several modes of entry. These can be termed, passive diffusion, facilitated diffusion and transport coupled to either electrochemical ion gradients or chemical transformations (Scarborough, 1985).

Passive transport can be rigorously defined as transport in which the system loses free energy (Lehninger, 1975) and the simplest case of this is free diffusion, namely the movement of a solute down a concentration gradient whereby the rate of movement is determined by, and proportional to, the difference in concentration (Jennings, 1963). As this is a physico-chemical process, temperature would be expected to influence the rate of diffusion as would the permeability of the membrane to the solute (Robertson, 1983). A more complex situation exists in facilitated diffusion. Scarborough (1985) defines facilitated diffusion systems as non-accumulative transporters that require only thermal energy for their operation. These transporters involve translocation down a concentration gradient mediated by a mobile membrane component, called a carrier. The carriers exhibit substrate specificity and the rate of solute transport is many times greater than by passive diffusion alone, exhibiting saturation kinetics at high solute concentrations (Robertson, 1983). When charged species are translocated by diffusion the movement is dependent not only on the concentration gradient, but also on the electric potential difference which arises when positive and negative charges across the membrane are imbalanced or are not moving at the same rate (Robertson, 1983).

A number of reports have detailed the passive transport of metals in microbes. Broda (1972) reported energy-independent uptake of lead in *Saccharomyces cerevisiae* and lead and cerium in the alga *Chlorella fusca*. Copper uptake in the fungus *Dactylium dendroides* appears to be a diffusion-limited process, the concentration gradient being maintained by rapid sequestering to intracellular components (Shatzman & Kosman, 1978) whilst Puckett *et al* (1973) proposed that passive absorption of divalent cations in a variety of lichen species occurred by way of a modified ion-exchange process involving the release of protons. Copper uptake by conidia of *Neurospora crassa* (Somers, 1963) and *Penicillium spinulosum* (Townsley & Ross, 1985) was unaffected by a number of metabolic inhibitors and determinations of Q_{10} values and the activation energy indicated passive uptake. However it has been argued that the inability to demonstrate energy-dependent transport in these studies may be due to the high wall binding of filamentous fungi masking low rates of intracellular influx (Gadd & White, 1985). In addition, it has been suggested that Co^{2+} uptake by *N.crassa* is a facilitated diffusion process (Venkateswerlu & Sastry, 1970) although Jasper and Silver (1977) have reinterpreted their findings as being the active transport of Co^{2+} by a system which is primarily responsible for Mg^{2+} uptake. It seems most unlikely that examples of truly passive diffusion in microbial inorganic ion systems exist (Silver, 1978). The microbial environment, unlike that of animal cells, is often highly dynamic and given widely fluctuating growth conditions it is unlikely that passive diffusion systems would place a microorganism at a competitive advantage. Indeed it is more likely that a cell with a high affinity system would have a selective growth advantage under limiting nutrient conditions (Silver, 1978). Metal retention by cells would also be complicated by diffusion back into a very dilute

aqueous environment.

A great many transport systems require a coupled input of free energy. Such systems often have to translocate a variety of small molecules and ions against a concentration gradient. To accomplish this, energy derived from metabolic activity must be applied to transport work. The mechanisms of energy coupling to membrane transport have still not been fully elucidated, although Mitchell's chemiosmotic hypothesis, its main tenets now generally accepted, has done much to clarify current thinking [for an excellent review of the role of chemiosmotic coupling in microbial transport, see Rosen and Kashket, (1978)]. By this hypothesis, the driving force of substrate uptake is provided by a transmembrane electrochemical ion gradient, which in turn, is established by systems which have been termed, primary active transport systems (Christensen, 1975, Rosen & Kashket, 1978). These are mechanisms for the conversion of chemical energy into electro-osmotic energy and examples of these are the H^+-ATPase of mitochondria and bacteria, the Ca^{2+} translocating ATPases of eukaryotic and prokaryotic systems, the K^+-ATPase of *Escherichia coli* and the Na^+/K^+-ATPase of animal cell plasma membranes (Scarborough, 1985). All these primary pumps depend upon the metabolism of the cell and generate transmembrane gradients of H^+, Na^+, K^+ and Ca^{2+} in a variety of cell types. Whilst primary pumps are examples of transporters coupled to chemical transformations (that is as a direct result of energy provided by a chemical reaction); secondary active transport systems are transporters coupled to electrochemical ion gradients (Rosen & Kashket, 1978). Thus, the ion gradients generated by primary systems are in turn used by specific ion-gradient-coupled transporters to drive nutrient accumulation (see Fig. 5.1.). When two substrates are transported simultaneously in one direction by one

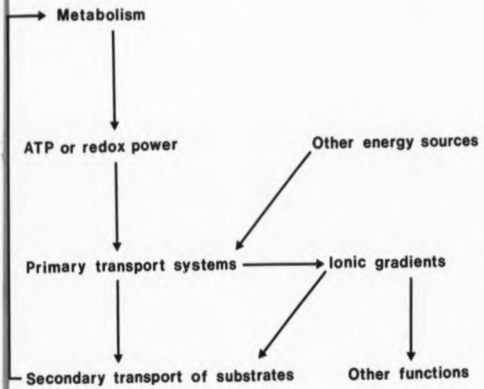


Figure 5.1. The energy requirement for transport

The diagram attempts to summarise how ion transport, by establishing ionic gradients, can be directly used as a form of energy to drive other transport systems. In addition, ion transporting systems are capable of stimulating metabolism to satisfy their energy requirement (Pena, 1974).

carrier, driven by the electrochemical gradient of one of the substrates, the process is known as symport. Conversely, when accumulation occurs on the side of the high electropotential of the driving ion, the process is known as antiport (Scarborough, 1985). In bacteria, a H^+ gradient usually provides the driving force whilst a Na^+ gradient generally drives secondary transport in animal cells (Stryer, 1981). All current findings suggest that the fungal plasma membrane ATPase is a primary H^+ pump, supplying energy for H^+-dependent nutrient cotransport (Goffeau & Slayman, 1981).

In yeasts and fungi a series of secondary transport systems have been identified. Uptake of nitrate in *C.utilis* (Eddy & Hopkins, 1985), glucose in *N.crassa* (Slayman & Slayman, 1974), amino acids in *S.cerevisiae* (Eddy *et al*, 1970a, Eddy *et al*, 1970b) and amino acids and nucleosides in *Schizosaccharomyces pombe* (Foury & Goffeau, 1975) all occur *via* proton symport systems. Yeast phosphate and sulphate transport is also thought to be driven by a proton gradient (Borst-Pauwels, 1981).

For the purposes of studying energy coupling to metal ion transport, the transmembrane electrochemical ion gradient, or proton motive force (pmf) as it is known when the primary electrogenic transporter translocates H^+ ions from the cells such as in fungi and bacteria, can be divided into two components; the electrical membrane potential difference across the lipid membrane and the difference in pH values on either side of the membrane (Goffeau & Slayman, 1981). In turn, the membrane potential may have several components; a) the Donnan potential resulting from indiffusible anions; b) the ion asymmetry created by ion pumps and the consequent potential resulting from different rates of diffusion out of the cells; c) the separation of

charge caused by primary electrogenic ion pumps (Clarkson, 1974). Each of these three components is dependent on cell metabolism. Until recently it was thought that monovalent cation uptake in yeasts was driven by the membrane potential (Riemersma & Alsbach, 1974), however Borst-Pauwels (1981) has suggested that K^+ influx occurs either by a primary plasma membrane ATPase system which pumps K^+ into the cell and H^+ out of the cell at the expense of ATP hydrolysis, or by using the pmf as the driving force.

A number of studies of divalent cation accumulation in bacteria, algae and plants have described an energy-dependent component of uptake (Broda, 1972, Ramani & Kannan, 1975, Silver, 1978). Similarly, the majority of yeast studies have reported the requirement for cellular energy in metal uptake (Borst-Pauwels, 1981). One of the earliest reports of cation absorption by Rothstein *et al* (1958) outlined the active transport of Mg^{2+} and Mn^{2+} in baker's yeast. A suitable energy source such as glucose is a prerequisite to uptake of divalent cations (Rothstein, 1958, Fuhrmann & Rothstein, 1968, Ross, 1977). Energy from either glycolysis or oxidative phosphorylation can drive Mn^{2+} transport in *Saccharomyces* species (Okorokov *et al*, 1977). Uptake of Ca^{2+}, Ni^{2+}, Co^{2+} or Zn^{2+} can use either fermentative or respiratory energy (Fuhrmann & Rothstein, 1968, Boutry *et al*, 1977) whilst Lichko *et al* (1980) reported that an ATPase is in some way involved in Mn^{2+} transport. The exact mechanism of energy coupling to divalent cation transport is still unclear although several authors have reported a second type of transport linked to a chemical transformation. Paton and Budd (1972) proposed that Zn^{2+} initially bound to polyphosphate in the surface membrane and then was released during transport by enzymic breakdown of this compound, which provided the energy for

translocation. A similar mechanism has been proposed for glucose uptake in yeast (Van Steveninck & Booij, 1964) whilst Jennings *et al* (1958) and Rothstein *et al* (1958) have postulated that a phosphorylated product is involved in the transport of divalent cations through the cell membrane. Whilst these mechanisms are not precluded, it seems likely that, in the light of Mitchell's chemiosmotic model, the electrochemical gradient, which effects transport of many other ions and small molecules, may play a major role and this subject will be discussed later.

In this section the surface binding and the energy requirement for transport of the metals, Mn^{2+} and Cu^{2+}, is investigated. Uptake of Mn^{2+} from both high and trace concentrations of Mn^{2+} is examined in view of the possibility that more than one transport system is being studied, as will become clear in the following chapter.

5.2. Materials and Methods

Exponentially growing cells were harvested and resuspended in MES buffer either with or without 20 mM glucose as stated. Metal uptake experiments and metal analyses were carried out as described in Chapter 3, with the inhibitors, carbonyl cyanide m-chlorophenylhydrazone (CCCP), sodium arsenate and 2,4-dinitrophenol (DNP), the ionophore, valinomycin, and the glucose analogue, 2-deoxyglucose, being added as required 15 min prior to the addition of Mn^{2+} or Cu^{2+}. The temperature was held at 30 ^{o}C or, where stated, 4 ^{o}C. CCCP, valinomycin and 2-deoxyglucose were obtained from the Sigma Chemical Co.

As previously mentioned, the cell density was adjusted such that only a small proportion of the total metal added was taken up by cells during an assay. In the case of accumulation from 50 μM Mn^{2+} or from 10 μM Cu^{2+}, the cell density was approximately 0.5 mg ml^{-1} and the proportion taken up after 20 min remained low, being 4 % and 6 % of the total metal added respectively. However, as the total metal is reduced, the proportion of metal accumulated for a given cell density increases considerably. Hence when uptake from a metal concentration as low as 10 nM is studied, the cell density of the incubation suspension becomes an important factor. When uptake conditions are optimised, there will be no significant change in the free metal concentration during the period of measured accumulation. Consequently, for experiments involving ^{54}Mn uptake, a preliminary study of the effect of cell density on the ^{54}Mn accumulated as a proportion of the total ^{54}Mn added is necessary. The results of such a study are shown in Table 5.2. It can be seen that the percentage of ^{54}Mn accumulation increased disproportionately to the increase in cell density and that at cell concentrations greater than 0.16 mg ml^{-1} the proportion bound was unacceptably large. Using this data, a final cell density of approximately 0.1 mg ml^{-1} was used in studies of ^{54}Mn accumulation.

5.3. Results

The effects of glucose and temperature on Mn^{2+} uptake from 50 μM Mn^{2+} are shown in Figure 5.3.1. It can be seen that in the presence of glucose, cellular Mn^{2+} increased in a fairly linear fashion over a period of 20 min. In the absence of glucose, in the presence of 2-deoxyglucose and at 4 ^{0}C, Mn^{2+} uptake was completely depressed with

Table 5.2. Effect of cell density on the accumulation of ^{54}Mn, as a proportion of total metal added

Cell density (mg ml^{-1})	^{54}Mn accumulation (% of total ^{54}Mn added)
0.03	0.6
0.08	1.0
0.16	7.5
0.30	31.5
0.55	50.0

Values given are for accumulation following 20 min incubation in 10 nM ^{54}Mn and represent the means of three experiments.

little or no diffusion into the cells occurring. To further test the involvement of glucose catabolism in providing energy for Mn^{2+} uptake, the metabolic poisons, CCCP, DNP and sodium arsenate were added to the incubation medium (Fig. 5.3.2.). Uptake of the metal was seen only in the absence of these inhibitors. The low level of Mn^{2+} associated with the cells in the absence of glucose is most likely to be due to non-specific and reversible binding to anionic groups on the cell wall. The slightly reduced binding seen at 4 ^{o}C would be as expected from this physical process.

^{54}Mn uptake from 10 nM Mn^{2+} proceeded almost linearly at 30 ^{o}C in the presence of glucose (Fig. 5.3.3.). However, at 4 ^{o}C or in the absence of glucose, uptake was not observed. Similarly, in the presence of CCCP and DNP no uptake occurred. Thus it appears that Mn^{2+} transport from either 50 μM or 10 nM Mn^{2+} is an energy-requiring process. Valinomycin, at concentrations up to 100 μM and at external K^{+} ranging from zero to 500 mM, exerted no demonstrable effect whatsoever on ^{54}Mn uptake. Figure 5.3.4. describes the uptake the uptake of Cu^{2+} from a 10 μM Cu^{2+} solution under conditions identical to those previously mentioned for Mn^{2+} uptake. Cu^{2+} uptake in the presence of glucose occurred rapidly over the first 5 min followed by a more linear uptake with a decreased rate. No Cu^{2+} uptake was observed in the absence of glucose, at 4 ^{o}C in the presence of glucose and in the presence of 2-deoxyglucose. However in the presence of the same concentrations of inhibitors as before, a different response was seen (Fig. 5.3.5.). Cu^{2+} transport was completely inhibited by DNP yet exhibited a greatly reduced response to CCCP and, to a lesser extent, to sodium arsenate than did Mn^{2+} transport from solutions containing either 50 μM or 10 nM Mn^{2+}.

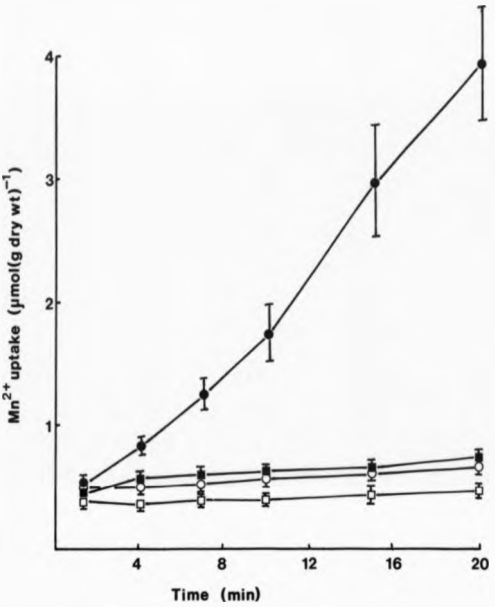


Figure 5.3.1. Mn^{2+} accumulation from 50 μM Mn^{2+} in the presence (●) or absence (■) of glucose; in the absence of glucose and the presence of 2-deoxyglucose (○); at 4 °C in the presence of glucose (□). Values represent the mean ± SE of 3 experiments.

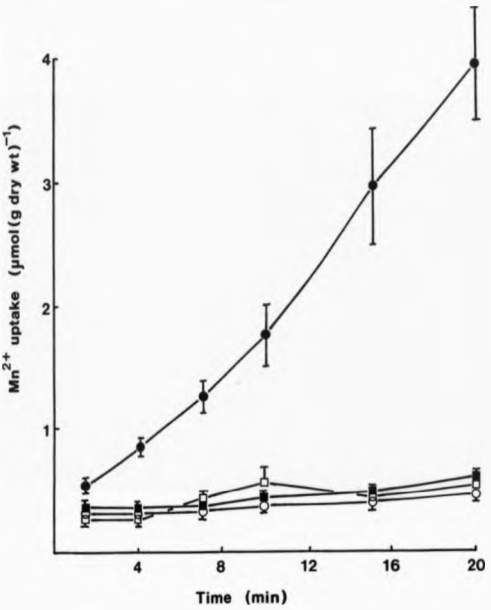


Figure 5.3.2. Mn^{2+} accumulation from 50 μM Mn^{2+} in the absence of inhibitor (●) and in the presence of 100 μM CCCP (■); 10 mM sodium arsenate (□); 2 mM DNP (○). Values represent the mean ± SE of 3 experiments.

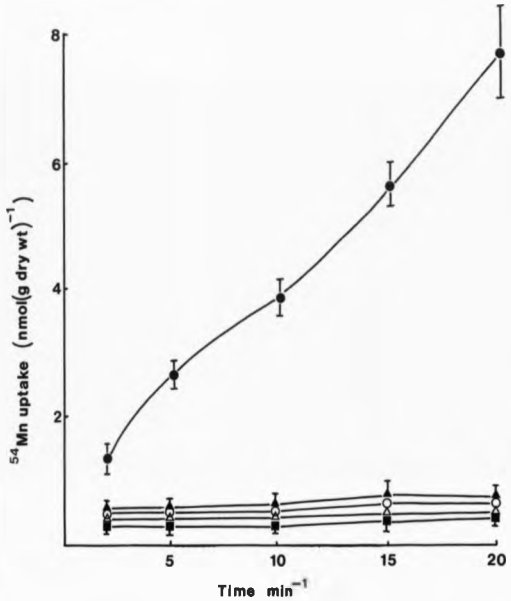


Figure 5.3.3. ^{54}Mn accumulation from 10 nM Mn^{2+} in the presence (●) and absence (△) of glucose; at 4 ^{o}C (▲) and in the presence of 100 μM CCCP (■) and 2 mM DNP (○). Values shown are the mean ± SE of 3 experiments (for clarity, only the maximum standard error bars have been included for uptake in the absence of glucose; at 4 ^{o}C and in the presence of CCCP and DNP).

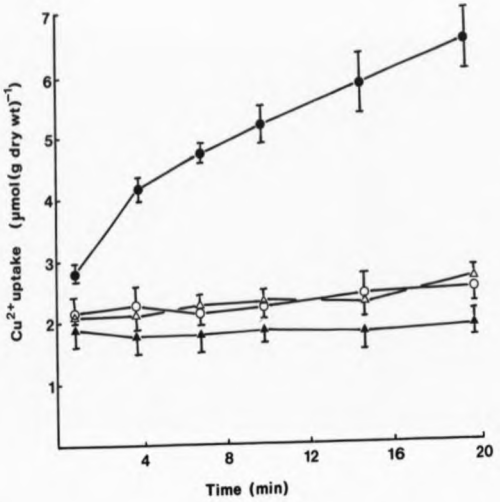


Figure 5.3.4. Cu^{2+} accumulation from 10 μM Cu^{2+} in the presence (●) or absence (○) of glucose; in the absence of glucose and the presence of 2-deoxyglucose (△); at 4 °C in the presence of glucose (▲). Values shown are the mean ± SE of 3 experiments.

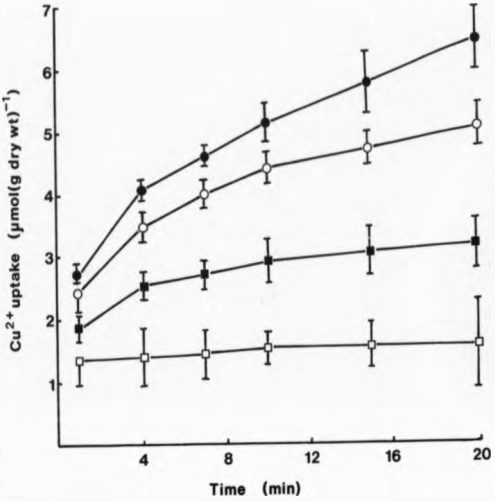


Figure 5.3.5. Cu^{2+} accumulation from 10 μM Cu^{2+} in the absence of inhibitor (●) and in the presence of 100 μM CCCP (○); 10 mM sodium arsenate (■); 2 mM DNP (□). Values represent the mean ± SE of 3 experiments.

Non-specific binding of the metal to anionic species on the cell surfaces occurred almost instantaneously. On a molar basis, comparatively more Cu^{2+} was bound to the cell surfaces; after 20 min in the presence of glucose, 39 % of the total Cu^{2+} taken up by cells was surface bound, as opposed to 19.1 % in the case of Mn^{2+} following incubation in 50 μM Mn^{2+}. Only 7.2 % of the total cell ^{54}Mn was associated with the surfaces of cells incubated for 20 min in 10 nM ^{54}Mn and this may well reflect the efficacy of the cell washing procedure used. Cells incubated in 10 nM ^{54}Mn were washed with 100 μM non-radioactive Mn^{2+} whilst cells incubated in 50 μM Mn^{2+} and 10 μM Cu^{2+} were washed with 2 mM $CaCl_2$. It seems likely that washing cells using the non-radioactive form of the metal under investigation is a much more efficient technique than the use of a second cation, such as Ca^{2+} or Mg^{2+}, given that 100 μM non-radioactive $MnCl_2$ proved much more effective at exchanging surface bound ^{54}Mn than did the use of 2 mM $CaCl_2$. Clearly, for the purposes of examining metal transport, this externally bound metal must be minimised, although obviously the use of 100 μM $MnCl_2$ wash solution was restricted in this present investigation to studies of ^{54}Mn uptake.

5.4. Discussion

Accumulation of Mn^{2+} and Cu^{2+} in cells of *C.utilis* appears to consist of two components; an initial rapid and reversible binding which is energy-independent and most probably represents binding to the cell surface, followed by a slower uptake into the cell which is metabolism-dependent. This finding is consistent with a number of other studies which have similarly demonstrated two phases of accumulation in yeast, a surface binding phase followed by an

energy-dependent influx (Failla *et al*, 1976, Norris & Kelly, 1977, Borst-Pauwels, 1981).

Although the surface binding is reduced in this study by the use of cation-exchange wash solutions, nevertheless, the avidity of surface sites for Mn^{2+} and, in particular, for Cu^{2+} ions is evident. The precise nature of the wall binding sites in *C.utilis* is unclear. Egorenkova and Belov (1984) have shown the walls of several strains of *Candida* species grown on a glucose carbon source to be composed of 31.4 mg g^{-1} biomass glucan and mannan and 3.0 mg g^{-1} biomass protein. In addition to mannan-protein and glucan-protein complexes, glucosamine and several enzymic proteins, such as invertase, are present in walls of *C.utilis* and *S.cerevisiae* (Rose, 1965). Hence, a variety of anionic binding sites will be available to Cu^{2+} and Mn^{2+} present at the cell surface. Surface binding of Co^{2+} from 3.4 mM Co^{2+} solution accounts for 30 % of total Co^{2+} accumulation in *N.crassa* (Venkateswerlu & Sastry, 1970) and 25 % of Mn^{2+} accumulation in baker's yeast was due to binding to the cell surface following incubation in 0.3 mM Mn^{2+} (Rothstein, 1958). Clearly, these values are only of limited use in comparing binding as the use of different experimental treatments will considerably affect the ratio of wall-bound metal to internalised metal. However in the present study, the greater affinity of Cu^{2+} for binding sites can be seen and this is supported by the data of Gadd and Mowll (1985) which showed that, of the seven other divalent cations studied, none were found to greatly affect surface binding of Cu^{2+} in *A.pullulans* when added in equimolar amounts to the copper. For a comparative analysis of binding in different yeast species, adsorption isotherms of the type mentioned in Section 5.1. can be constructed using the binding data although, in this case, as the work mainly concerns intracellular

influx, isotherms have not been applied.

The relationship between surface binding and uptake is not clear. Townsley and Ross (1985) have suggested that the initial binding may serve as a scavenging mechanism whereby a high localised concentration of metal results in the vicinity of the cell wall or membrane. Release of these ions from their bound locality, probably by H^+ ions, may result in subsequent internalisation. This postulate is supported by the data of Paton and Budd (1972), who proposed that surface binding is a prerequisite for transport into the cell. However, evidence from Gadd and Mowll (1985) that Ca^{2+} reduced surface Cu^{2+} binding but did not affect rates of Cu^{2+} influx, indicate that complete surface binding is not necessarily a prerequisite for intracellular Cu^{2+} transport in *A.pullulans*.

The differing shape of the uptake profiles of Mn^{2+} and Cu^{2+} is of interest. Mn^{2+} uptake follows a marked linear pattern whilst the Cu^{2+} uptake rate was consistently greater during the initial 5 - 10 min of exposure to the metal. This reduction in the transport rate during later stages of uptake is difficult to explain although this phenomenon has been reported by several other authors (Norris & Kelly, 1977, Gadd *et al*, 1984a, Wakatsuki *et al*, 1985). It is conceivable that when the intracellular Cu^{2+} concentration reaches a certain threshold, slight toxic effects result, manifested by a reduction in uptake rate. This may be due to Cu^{2+} ions binding to structural or enzymic proteins involved in transport or metabolism. The likelihood of this appears greater when it is noted that a small reduction in respiration was observed following incubation in 10 μM Cu^{2+} (see Section 4.3.2.).

In the system used for ^{54}Mn uptake, at a cell density of 10^7 cells ml^{-1}, occupying a packed cell volume of approximately 0.1 % (v/v) of the medium [assuming intercellular spaces to occupy around 22 % of the total volume of the packed cells, as determined by Conway and Downey (1950)], 10^7 cells took up 1.53×10^{-3} nmol during 20 min incubation in 10 nM ^{54}Mn. Assuming all cellular ^{54}Mn to be in an unbound, ionic form, this represents an uphill transport concentration gradient of approximately 180:1 (inside:outside). The concentration gradient for cellular Cu^{2+} following 20 min uptake from 10 μM Cu^{2+} was approximately 90:1 (inside:outside) whilst a value of approximately 15:1 (inside:outside) was observed in cells following 20 min incubation in 50 μM Mn^{2+}. These values have been corrected to take account of surface binding and so give a more accurate indication of the ^{54}Mn, Mn^{2+} and Cu^{2+} actually internalised.

Micro-organisms are known to be capable of concentrating metal ions present in low concentrations in the external milieu and enrichment factors (ratio of concentration in cell and medium) as high as 10^6 have been reported (Broda, 1972, Aickin & Dean, 1978). In a study of several yeast and fungal species, Okorokov *et al* (1975) reported that Mn^{2+} was taken up against concentration gradients of 25:1 to 1300:1 and suggested that it may be assumed that an active transport system was in operation. Zn^{2+} uptake in *C.utilis* is against a concentration factor of approximately 125 (Failla *et al*, 1976) whilst in *N.crassa*, Co^{2+} is concentrated only to a small extent within the mycelia (an intracellular concentration of 11 - 13 mM Co^{2+} at 10.2 mM external Co^{2+}) (Venkateswerlu & Sastry, 1970). Jasper and Silver (1977) have subsequently proposed that Co^{2+} uptake in *N.crassa* is *via* a Mg^{2+} transport system and that this latter low value would be due to the low affinity of the Mg^{2+}-transporter for Co^{2+}. It is now thought that a similar situation exists in *C.utilis* with Mn^{2+} transport

occurring by way of a specific Mn^{2+} uptake system at low Mn^{2+} concentrations and *via* the Mg^{2+}-transporter at high Mn^{2+} concentrations (see Chapters 6 & 7) and this may, in part, explain the rather low concentration factor observed following incubation in 50 μM Mn^{2+} as compared to the value obtained using 10 nM ^{54}Mn. This difference may also be due to the non-physiological high external Mn^{2+} concentration tested. Indeed the total cellular metal content of micro-organisms appears to remain relatively constant even as the external concentration increases greatly (Okorokov *et al*, 1975, Aiking *et al*, 1985) and in the bacterium, *E.coli*, cellular Mg^{2+} is generally in the narrow range of 15 - 35 mmol Kg^{-1} wet cells, even when the growth medium range varies by a factor of 100,000 (Jasper & Silver, 1977).

The use of concentration factors makes the assumption that all the cellular metal is present in an osmotically free form. This is unlikely to be the case, Okorokov *et al* (1977) estimated that 78 % of the total Mn^{2+} was bound to cellular components of *Saccharomyces carlsbergensis* whilst other estimations of bound metal vary widely, depending on the species and growth conditions, from 28 % to 98 % of the total cellular metal (Rothstein & Hayes, 1956, Venkateswerlu & Sastry, 1970, Okorokov *et al*, 1975). Owing to redistribution problems and artifacts occurring during cell fractionation, the nature of the binding sites is difficult to determine. 55 % of intracellular Zn^{2+} was released following exposure of cells of *C.utilis* to organic solvents or nystatin, indicating that some of the Zn^{2+} is free or may be bound to low molecular weight substances such as amino acids or nucleotides (Failla *et al*, 1976). A further 30 % of the Zn^{2+} is released on heating cells to 100 ^{o}C, suggesting that some Zn^{2+} is also bound to macromolecules that are denatured by heat.

Up to 50 % of cellular bivalent cations are bound to non-diffusible anions such as proteins and nucleic acids (Rothstein & Hayes, 1956). Other authors report that bivalent cations may be compartmentalised within cell vacuoles and sequestered to ribosomal material, polymeric orthophosphate compounds or a series of soluble, sulphur-rich metal binding proteins, collectively referred to as metallothioneins (Failla, 1977, Jasper & Silver, 1977, Borst-Pauwels, 1981).

Dependence upon a cellular energy source is a fairly well documented feature of metal cation transport systems in micro-organisms (Silver, 1978, Borst-Pauwels, 1981). Energy-dependent uptake of Mn^{2+} (Rothstein *et al*, 1958, Okorokov *et al*, 1977, Bianchi *et al*, 1981) and Cu^{2+} (Ross, 1977, Gadd *et al*, 1984a) has been observed in the yeast, *S.cerevisiae*. The results clearly demonstrate the transport of both metals in *C.utilis* to be metabolism-dependent. The absence of any uptake of metal ions when 2-deoxyglucose is substituted in place of glucose implies no interaction with the glucose transport system, since 2-deoxyglucose is transported and phosphorylated by yeast but not further metabolised (Van Steveninck, 1968). The lack of diffusion into the cells in the absence of glucose indicates little permeability of the cell membrane for the two cations. Mn^{2+} and Cu^{2+} transport was completely dependent on the presence of glucose, Bianchi *et al* (1981) suggest that this result indicates a relation between transport and the activity of energy-dependent electrogenic pumps. This result is in accordance with a number of other reports, Zn^{2+} uptake in *C.utilis* shows a lag time of 16 min after the addition of glucose (Failla *et al*, 1976) and the stimulatory effect of glucose on metal transport has been widely documented (Borst-Pauwels, 1981). The lack of Mn^{2+} influx in the presence of the uncoupling agents, DNP and CCCP (Heytler & Pritchard,

1962, Harold *et al*, 1974) and the inhibitor of substrate level phosphorylation, sodium arsenate (Stryer, 1981, Archibald & Duong, 1984) is indicative of a metabolically dependent process. The apparent reduced sensitivity of copper uptake, as compared to manganese, to CCCP and arsenate inhibition is somewhat difficult to interpret. Earlier reports of cation transport inhibition using CCCP (Silver *et al*, 1970, Failla & Weinberg, 1977) have demonstrated complete suppression of metal uptake. However, the effect on Cu^{2+} uptake was not previously studied. A recent study by Laddaga *et al* (1985) demonstrated a Cd^{2+}-resistant mutant of *Bacillus subtilis* in which Mn^{2+} transport, but not Cd^{2+} transport, was sensitive to CCCP. In wild-type cells, transport of both metals was sensitive to CCCP. No explanation for this effect was given.

The concentration of inhibitors used in the present study is as used in a number of previous studies of metal uptake. CCCP at 100 μM has been used to effectively inhibit Zn^{2+} uptake in *C.utilis* (Failla & Weinberg, 1977, Lawford *et al*, 1980), uptake of Mn^{2+}, Ca^{2+}, Cd^{2+} and Co^{2+} in *S.cerevisiae* and *S.carlsbergensis* (Carafoli *et al*, 1970, Norris & Kelly, 1977, Lichko *et al*, 1980) and Mn^{2+} in bacterial species (Eisenstadt *et al*, 1973, Archibald & Duong, 1984). Sodium arsenate and DNP have also been used at identical concentrations to those used in this study to inhibit Mn^{2+} uptake in yeast and bacteria (Lichko *et al*, 1980, Archibald & Duong, 1984). In view of the fact that, at 2 mM, the DNP concentration is somewhat higher than the optimum required to cause maximum proton influx into yeast cells [0.5 mM DNP (Gadd & Mowll, 1985)], the possibility that other inhibitory effects are exerted is not precluded. DNP is taken up by yeast cells (Kiesow, 1959) and also inhibits glucose uptake (Kotyk & Janacek, 1975) and may exert a number of other disruptive effects on

cellular metabolism, therefore conclusions on the basis of the results of DNP alone acting as an uncoupler should be viewed with care. This cautionary note, regarding the interpretation of DNP sensitivity at DNP concentrations greater than those necessary to uncouple phosphorylation has been emphasised by Jennings (1963). Despite these reservations, this study shows that both Mn^{2+} and Cu^{2+} uptake are energy-requiring. Proton conductors do not inhibit transport processes directly but dissociate them from the metabolic machinery by allowing the free entry of H^{+}, thus collapsing the pmf (Harold *et al*, 1974), depolarising the plasma membrane and abolishing the membrane potential (Gadd & Mowll, 1985). As metal influx is inhibited by CCCP and DNP, this indicates that Mn^{2+} and Cu^{2+} transport may be driven by the membrane potential. It is possible that the different response to CCCP seen in Mn^{2+} and Cu^{2+} uptake indicates that Cu^{2+} transport is coupled to an energy input in a slightly different manner to that of the Mn^{2+} transport system. One possible explanation is that Cu^{2+} transport is not as greatly dependent upon a plasma-membrane proton gradient.

The involvement of a plasma-membrane ATPase in yeast divalent cation transport has been suggested (Fuhrmann & Rothstein, 1968). The initial rate of divalent cation uptake is directly proportional to the ATP content at the beginning of the uptake experiment and during uptake, ATP is partially used up (Fuhrmann, 1974a). In addition, the same sequence is found for the stimulation of membrane ATPase by divalent cations as the order of affinity for the uptake of the divalent cations, and membrane ATPase inhibitors completely inhibit cation uptake (Fuhrmann, 1973, 1974b). These results indicate that a membrane-bound transport ATPase may be directly involved in divalent cation influx. Subsequent studies in *S.cerevisiae* have shown that

there is no detectable selectivity in the initial uptake of Mn^{2+} and Sr^{2+} whilst the plasma-membrane ATPase is specific for Mn^{2+} yet not for Sr^{2+} (Peters & Borst-Pauwels, 1979, Nieuwenhuis *et al*, 1981). Hence a direct role of the yeast membrane ATPase in divalent cation uptake can be ruled out although it is likely that the membrane ATPase is involved in the generation of an electrogenic potential. Roomans *et al* (1979) and Bianchi *et al* (1981) have shown that Ca^{2+}, Sr^{2+}, Mn^{2+} and Mg^{2+} uptake may be coupled to proton efflux or may depend on the membrane potential generated by a proton pump. H^+-ATPase systems are responsible for the transmembrane proton gradient which drives Ca^{2+} / H^+ antiport in *S.cerevisiae* (Eilam *et al*, 1985a), *N.crassa* (Stroobant & Scarborough, 1979) and in *B.subtilis* (de Vrij *et al*, 1985), and in yeast membrane vesicles, Mn^{2+} uptake was driven by a proton gradient (Borst-Pauwels, 1981). As an additional driving source for divalent cation transport, a K^+ gradient has been proposed (Lichko *et al*, 1980). This idea is supported by studies using ethidium bromide which promotes an electrogenic K^+ efflux which, in turn, generates an increased negative potential within the cell, stimulating Ca^{2+} and Mn^{2+} influx (Pena, 1978). Thus, there are indications that divalent cation uptake in yeasts is driven by the membrane potential (Borst-Pauwels, 1981, Gadd & Mowll, 1985) and this may be the case in this study for Mn^{2+} and Cu^{2+} uptake in *C.utilis*. Indeed, further evidence for this is the inhibitory effect of high external K^+ concentrations which may result in membrane depolarisation due to high intracellular K^+ accumulation and also the involvement of an outward movement of protons concomitant with Mn^{2+} influx and these effects are demonstrated and discussed in a later chapter.

The primary purpose of the experiments outlined in this chapter was to establish energy dependence of Mn^{2+} and Cu^{2+} uptake. Clearly, this has been seen, however there is great scope for further studies employing more specific inhibitors (such as ionophores, to depress the membrane potential, or ATPase inhibitors), and the use of yeast plasma-membrane vesicles in transport studies in order to fully investigate the energy coupling to divalent cation influx. Indeed, the application of these techniques holds some promise, although in practise they may be fraught with difficulties. Of the ATPase inhibitors known to be effective in yeasts and fungi, only two, orthovanadate and diethylstilbestrol, are specific inhibitors of the plasma-membrane ATPase. The others are either specific for the mitochondrial ATPase or are non-specific (Goffeau & Slayman, 1981). Both compounds are useful for studies on purified plasma-membrane ATPase and in cell fractionation experiments yet are of limited use in whole-cell studies, as vanadate can inhibit or inactivate a wide range of other cellular enzyme systems (Macara, 1980) whilst diethylstilbestrol is known to block at least five other distinct transport systems in fibroblasts (Goffeau & Slayman, 1981) and can also disrupt lipid bilayers (Weissmann *et al*, 1976).

Ionophores have been used with great effect to determine the energy coupling for various transport systems in bacterial cells and vesicles (Harold *et al*, 1974, Rosen & Kashket, 1978) however their efficacy seems greatly reduced in whole-cell yeast studies and this may, in part, be due to limitations on diffusion exerted by the rigid cell wall structure (Theuvenet & Bindels, 1980). Valinomycin, a K^+ specific ionophore which allows K^+ to diffuse through the membrane down a concentration gradient thus affecting the membrane potential (Harold *et al*, 1974), has little effect on yeasts when compared with

bacteria (Gadd & Mowll, 1985) and, at a concentration of 100 μM, promotes only a slight movement of K^+ in *A.pullulans* cells (Mowll & Gadd, 1984). In this study of *C.utilis*, valinomycin appeared to have no effect on metal uptake. One answer to this problem may be the use of yeast protoplasts, in which some transport properties are more sensitive to ionophore antibiotics [for example, the effectiveness of the antibiotic, Dio-9, in causing net proton influx is at least fifty times higher after enzymic removal of the yeast cell wall (Theuvenet & Bindels, 1980)], or plasma-membrane vesicles (Fuhrmann *et al*, 1974) where diffusion of the ionophore into the plasma-membrane can occur uninhibited.

In conclusion, Mn^{2+} and Cu^{2+} accumulation in *C.utilis* has been shown to comprise an initial energy-independent binding to the external cellular surfaces followed by an intracellular influx which requires energy derived from metabolic processes and which is blocked by low temperature, the absence of a catabolite source and by a number of metabolic inhibitors. In the next chapter, the effect of other divalent cations on the uptake of Mn^{2+} and Cu^{2+} will be examined in order to investigate the specificity of their transport systems.

6. UPTAKE SPECIFICITY: THE EFFECT OF DIVALENT CATIONS ON MANGANESE AND COPPER TRANSPORT

6.1. Introduction

Of the 92 elements of the periodic table, around 30 have been found to be required for microbial growth, the requirement varying from species to species. Non-essential elements are generally of low abundance and should provide little competition with essential elements for cell sites (Wood, 1984a) nevertheless, cells are capable of exhibiting a high discrimination for a number of essential metal ions. The effects of competing cations on microbial metal uptake have attracted a great deal of attention and much of the recent work has been reviewed by Gadd and Griffiths (1978), whilst Borst-Pauwels (1981) has reviewed cation selectivity in yeasts. However, as will become clear in the succeeding text, there are a number of significant omissions and this is partly due to a lack of available data on uptake from low, physiological concentrations of metals. Indeed, in a discussion of the evolutionary aspects of metal ion transport, Wood (1984b) has noted the great lack of understanding of the selectivity principles involved in trace metal transport. Silver (1983) suggested that metals fall into three categories: a) essential nutrient ions comprising macronutrient minerals, such as Mg^{2+} and K^{+}, and trace or micronutrient minerals, including Mn^{2+}, Fe^{2+} and Zn^{2+} plus ions of possibly 20 other elements, for which the microbial cell has evolved transport systems to ensure an adequate supply for growth and division; b) unessential, but common or abundant cations, such as Na^{+}, the intracellular levels of which are also regulated by transport systems of the cellular membrane often *via* outwardly orientated transporters that maintain the intracellular concentration

well below that of the external milieu; c) toxic ions of no known use for which the cell has not evolved transporters but which often exert their toxic action by way of fortuitous intracellular accumulation *via* the existing transport systems of other cations. The majority of reports and experimental data mentioned in this chapter will be pertinent to metal ions of the first category as it is these metals which are of physiological and evolutionary importance in terms of their transport systems.

Some of the first reports detailing divalent cation uptake in yeasts demonstrated that Mg^{2+} could be transported by a monovalent cation carrier which was primarily responsible for the physiological transport of K^+ ions (Conway & Beary, 1958). Mg^{2+} competed with a number of monovalent cations for binding to the same transport sites with the relative affinities for the cation carrier being: $H^+ > K^+ > Rb^+ >> Cs^+ > Na^+ >> Li^+$, Mg^{2+} (Conway & Duggan, 1958, Armstrong & Rothstein, 1964). Hence Mg^{2+} transport by this route occurred only when the Mg^{2+} ion was the only inorganic cation present in the external fluid in appreciable concentration. This system was also pH sensitive; below pH 5.5 Mg^{2+} influx was *via* a separate mechanism. Subsequent studies have shown that, at relatively low concentrations, Ca^{2+} and Mg^{2+} behave as non-competitive inhibitors of K^+ transport and this led to the proposal that two sites were involved in the uptake process, a transport or carrier site with a high affinity for K^+, and a modifier site which was thought to influence the maximal transport rate of the carrier system (Armstrong & Rothstein, 1967). In their review of microbial magnesium transport, Jasper and Silver (1977) preferred to attribute the effects of divalent cations on K^+ transport to more general and non-specific effects on the yeast cell surface. The fact that

inhibition of Zn^{2+}, Co^{2+}, Ni^{2+} and Ca^{2+} in yeasts by K^{+} is a non-competitive effect (Fuhrmann & Rothstein, 1968, Boutry *et al*, 1977) confirms that divalent cations are not transported *via* the monovalent cation carrier. Indeed, the inhibition of Ca^{2+} and Sr^{2+} uptake in *S.cerevisiae* by monovalent cations has been described as being due to a depolarising effect on the membrane potential rather than a truly non-competitive inhibition (Roomans *et al*, 1979). Borst-Pauwels (1981) concludes that the monovalent cation carrier is probably of minor significance for the provision of yeasts with necessary amounts of divalent cations.

Several early reports of divalent cation uptake from Rothstein's laboratory outlined a system in *S.cerevisiae* which preferentially translocated Mg^{2+} and Mn^{2+} as compared to Ca^{2+}, Sr^{2+} and UO_2^{2+} (Rothstein, 1958, Rothstein *et al*, 1958). It now appears from the available literature that in probably most, if not all, yeast species there exists a carrier of broad specificty for which magnesium is a primary substrate whilst other divalent cations, exhibiting lower affinities for the carrier, are also transported. There exists a wealth of evidence for this which is summarised here.

Fuhrmann and Rothstein (1968) produced an affinity series for the Mg^{2+} carrier of *S.cerevisiae* as follows:
Mg^{2+}, Co^{2+}, $Zn^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+}$. This was based on direct comparisons of Mn^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+} transport, on competition experiments between pairs of cations, on a common energy source from fermentation and the dependence on a phosphate pretreatment period. Mg^{2+} ions markedly inhibited the rate of Mn^{2+} uptake in both Mn^{2+}-sensitive and Mn^{2+}-resistant strains of *S.cerevisiae* (Bianchi *et al*, 1981). In the same species, Putrament *et al* (1977) showed

clearly that Mg^{2+} competition reduced Mn^{2+} uptake to a much greater extent than Zn^{2+} competition. They attributed the discrepancy between this data and the affinity series obtained by Fuhrmann & Rothstein (1968) to differing experimental conditions and the genetic properties of the strains tested. Mg^{2+} inhibited both uptake and binding of Ca^{2+} in yeast and a kinetic analysis agreed with the existence of a single transport system for both cations (Borbolla & Pena, 1980). Niewenhuis *et al* (1981) and Theuvenet *et al* (1986) demonstrated that, in fact, the divalent cation transporter probably exhibited very little selectivity for cations, as the previously reported higher specificity for Mn^{2+} as opposed to Sr^{2+} was due to a greater efflux of Sr^{2+} from the cells giving rise to an apparent faster influx of Mn^{2+}. The initial rates of entry into yeast cells was the same for both metals. Mg^{2+} and Ca^{2+} inhibited Mn^{2+} uptake by *S.cerevisiae*, Mg^{2+} being a more effective inhibitor than Ca^{2+} (Okorokov *et al*, 1979). Sr^{2+} was also found to competitively inhibit Ca^{2+} uptake (Roomans *et al*, 1979) and competition experiments revealed that inhibition of Co^{2+} uptake decreases down the following series:
$Zn^{2+} > Ni^{2+} > Mg^{2+} > Mn^{2+} > Cd^{2+} > Ca^{2+}$ (Norris & Kelly, 1977). This was related to the crystal ionic radii of the cations. Other reports in yeasts have shown that Co^{2+} and Cu^{2+} uptake in *Rhodotorula glutinis* is inhibited in Mg^{2+}- hypertonic medium (Joho, 1975). Ni^{2+} uptake was inhibited by Mg^{2+} more than by Zn^{2+} in *C.utilis* (Norris & Kelly, 1979), whilst Mn^{2+} counteracts Cd^{2+} and Cu^{2+} toxicity in *S.cerevisiae* (Sahinkaya, 1960) and Zn^{2+} and Mn^{2+} also decrease the toxic effects of high concentrations of Mg^{2+}, Cd^{2+} and Cu^{2+} in the same species (Jones & Greenfield, 1984).

Although reports of metal uptake in filamentous fungi are more fragmented, a similar common divalent cation carrier is also thought to be present. Paton and Budd (1972) showed that Mn^{2+} behaved as a competitive inhibitor of Zn^{2+} translocation whilst the effects of Mg^{2+} were of the 'mixed' type, increasing the apparent K_t and decreasing the rate of Zn^{2+} uptake. Cu^{2+}, Ni^{2+} and Co^{2+}, when present at the same molarity as Zn^{2+}, were also shown to have some inhibitory effect on Zn^{2+} uptake. Mg^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+} competed for Co^{2+} transport sites in *Neurospora crassa*, the effect being strain specific (Mohan & Sastry, 1984, Mohan *et al*, 1984) and it was suggested that the system that transports Mg^{2+} is also involved in Co^{2+} uptake in *N.crassa* (Venkateswerlu & Sastry, 1970). In addition, cells of *Penicillium ochro-chloron*, grown in media containing 1 g l^{-1} $MgSO_4$, were found to have a decreased Mg^{2+} cellular content when in the presence of various concentrations of Cd^{2+}, Zn^{2+} or Cu^{2+} (Okamoto *et al*, 1977).

If other bivalent trace elements are exclusively taken into cells simply as alternative low affinity substrates of the Mg^{2+} transporter, the problem arises as to how a cell might obtain essential elements such as Mn^{2+}, Zn^{2+} or Cu^{2+} when, as in normal growth medium, there is a great excess of Mg^{2+} (often up to 10,000-fold). Obviously, the cellular requirements for trace elements are much lower than for Mg^{2+} but nevertheless, the ability of the microbial cells to scavenge for these elements would be severely compromised under such conditions. The first report of a transport system which was specific for Mn^{2+} in the bacterium, *Escherichia coli*, appeared in 1969 (Silver & Kralovic, 1969). Since then, a number of studies of *E.coli* have unequivocally demonstrated, in addition to a non-specific Mg^{2+} transporter analogous to those

observed in yeasts (Webb, 1970, Nelson & Kennedy, 1971, Silver & Clark, 1971, Park *et al*, 1976), the presence of two separate transport systems for Mg^{2+} (Nelson & Kennedy, 1972, Park *et al*, 1976) and Mn^{2+} (Silver *et al*, 1970). These energy-linked transporters exhibited both high specificity and high affinity and were generally detected only in cells grown with very low metal concentrations, which presumably induce such pathways. Similarly, highly specific active transport systems for Mn^{2+}, which were unaffected by concentrations of other divalent cations several orders of magnitude greater than that of the Mn^{2+}, have been observed in *Bacillus* spp. (Silver & Jasper, 1977), *Rhodopseudomonas capsulata* (Jasper & Silver, 1978), *Staphylococcus aureus* (Perry & Silver, 1982) and *Lactobacillus plantarum* (Archibald & Duong, 1984).

As a result of his bacterial metal uptake studies, Silver formulated a unifying hypothesis for ion transport: for each and every inorganic mineral cation or anion that a cell requires for growth, separate highly specific transport systems exist (Silver, 1978, 1983). The relative physiological concentrations of metals and their availability in the external media will be reflected in the presence and specificity of the metal transport systems. The cell's macro-requirements, such as Mg^{2+}, are generally present at higher concentrations and are more readily available to cells, and thus a transporter of only low specificity but having a high rate of Mg^{2+} translocation is required. Minerals of low availability, such as Mn^{2+}, Cu^{2+} and Zn^{2+}, are only required in small amounts and hence the presence of transporters of high specificity for each ion would allow the cell to scavenge these metals, in preference to Mg^{2+}, from an environment containing only very dilute concentrations of these ions. It is worthy of mention that, in bacterial cells, in addition to the

general Mg^{2+} transporter, there also exists a highly selective Mg^{2+} scavenging system which is repressed during growth in 10 mM Mg^{2+} (Park *et al*, 1976) and only operates under conditions of Mg^{2+} stress to ensure that, whatever the external Mg^{2+} concentration, the cell still receives a sufficient supply of this important metal.

It can be predicted that energy-dependent transport systems specific for Mn^{2+} will be found in essentially all living cells (Silver & Jasper, 1977). Certainly, if other cell types are examined, there does seem some basis to this, Silver and Jasper (1977) reported specific Mn^{2+} transporters in *Euglena gracilis* and in human KB cells. Mn^{2+} transport in rice seedlings has been described (Ramani & Kannan, 1975) and Clarkson (1974) reported that the rate of Mn^{2+} influx in sugar-cane leaf tissue was unaffected by the presence of 0.1 mM Zn^{2+} or Cu^{2+}. Similarly, neither Cu^{2+} nor Zn^{2+} transport was affected by Mn^{2+} and all three uptake systems had similar K_t and V_{max} values. The question is therefore: why have there been no reports of specific Mn^{2+} transport in yeasts, despite the large number of uptake studies using Mn^{2+}? The answer may lie in the fact that previous studies of Mn^{2+} uptake in *Saccharomyces* spp. (Norris & Kelly, 1977, Okorokov *et al*, 1979, Nieuwenhuis *et al*, 1981) have generally used media containing relatively high Mn^{2+} concentrations (in the range 0.2 mM to 3 mM). Under these conditions saturation of specific high affinity transport systems may occur thus masking their detection. Hence previous authors, in studying the uptake of Mn^{2+} in yeasts, may well have observed Mn^{2+} transport *via* the low affinity Mg^{2+} transport system. It would not seem unreasonable to suppose that, at submicromolar concentrations of metals, these specific systems might be detected.

Gadd and White (1985) stressed that fungal cells must have influx mechanisms for entry of essential metal ions such as Cu^{2+} or Zn^{2+}. Indeed, work previously carried out in *C.utilis* suggests that Zn^{2+} transport occurs by way of a highly specific process (Failla *et al*, 1976) whilst data on *S.cerevisiae* suggest that it does not (Borst-Pauwels, 1981). This may indicate major differences in metal ion transport between the two species or may be a reflection of the very low Zn^{2+} concentrations used in the former study which might facilitate detection of specific metal transporters similar to those observed in bacterial species.

The hypothesis that each essential metal will have a specific transport system may well explain some reports of yeast metal ion uptake, notably that uptake of Ca^{2+}, Cu^{2+} and Cd^{2+} were clearly incompatible with the idea of a general transport mechanism whereby all divalent cations enter the cells as low affinity substrates of the Mg^{2+} carrier. Ca^{2+} uptake in *Schizosaccharomyces pombe* was *via* a high affinity system, however the specificity was not absolute as the system had lower affinities for $Sr^{2+} > Mn^{2+} > Co^{2+} > Mg^{2+}$ (Boutry *et al*, 1977). It seems unlikely that Ca^{2+} influx occurred by the Mg^{2+} transporter and it was observed that, at higher Ca^{2+} concentrations, a second Ca^{2+} carrier was detected and this may have been the Mg^{2+} uptake system. Gadd and Mowll (1984) observed that Cd^{2+} uptake in *Aureobasidium pullulans* was competitively inhibited by Ca^{2+} yet unaffected by Zn^{2+}, Co^{2+}, Hg^{2+}, Mn^{2+} and Cu^{2+}. Kessels *et al* (1985) also noted the reduction of Cd^{2+} uptake by Ca^{2+} whereas Mg^{2+} ions had no effect. Cu^{2+} transport in *A.pullulans* was completely unaffected by equimolar concentrations of Mg^{2+} yet was inhibited by (in decreasing order) $Mn^{2+} > Cd^{2+} > Zn^{2+} > Ca^{2+}$, Co^{2+} (Gadd and Mowll, 1985). They suggested that Cu^{2+} and Ca^{2+} were translocated by

different systems. In addition, Wakatsuki *et al* (1979) showed that 0.1 mM of salts of Mg^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+} had no effect on the uptake of Cu^{2+} following 60 min incubation in 0.1 mM Cu^{2+}. Zn^{2+} and Ca^{2+} only exerted 13 % reduction of Cu^{2+} uptake when present at 0.1 mM. All this data is consistent with the presence of fairly specific transport systems in these yeast species for Ca^{2+} and Cu^{2+}, with Cd^{2+} acting as a fortuitous inhibitor of the Ca^{2+} carrier. Finally, on a note of caution, Norris and Kelly (1979) have shown that the selectivity of the yeast cell for divalent cations depends upon the species of yeast and Borst-Pauwels (1981) has pointed out that due to the complexities of metal-yeast cell interactions, care should taken in concluding that there exists more than one translocation mechanism with different cation selectivities.

This section of the study is aimed at assessing two main tenets: whether or not there exists in *C.utilis* a common divalent cation carrier of broad specificity, and, if this is so, whether there also exist any highly specific systems for transport of the micronutrients, Mn^{2+} and Cu^{2+}, which would normally be saturated (and thus undetected) under conditions of high Mn^{2+} or Cu^{2+} concentrations. To determine the latter possibility, transport of the metal would have to be from media containing particularly low metal concentrations and for this purpose, the radionuclide ^{54}Mn was employed as was the use of the sensitive detection technique of anodic stripping voltammetry for Cu^{2+} determinations.

6.2. Materials and methods

The preliminary procedures and metal uptake experiments are as previously described in Chapter 3. All competing metals were added as chloride salts at 1 min before the addition of the metal under investigation. DNP was added 15 min prior to metal additions. Oxygen uptake experiments were carried out as described in Section 4.2.2. except that oxygen uptake was monitored from 6 min following the addition of metal salts. Where the mean and estimates of the variance of the mean of a set of data is obtained from percentages or proportions then it is statistically improper to determine these values directly. In these instances, the arcsine transformation of the data must be carried out and the mean and standard errors determined from the transformed data then back-transformed to give the familiar scale (Sokal & Rohlf, 1969). Hence, in all cases where data has been transformed in this manner in the present study, the mean and standard error range are given in the back-transformed scale. However, as transformed standard error values are asymmetrical, the range of the standard error is given as the upper and lower limits instead of the more familiar plus or minus S.E. notation.

6.3. Results

6.3.1. Effect of divalent cations on copper uptake

Figure 6.3.1. shows the time-course of Cu^{2+} uptake from 5 μM Cu^{2+} solution in the presence of 10-fold additions of various other divalent cations. Neither surface binding of Cu^{2+}, nor intracellular Cu^{2+} uptake was significantly affected, at the various time intervals

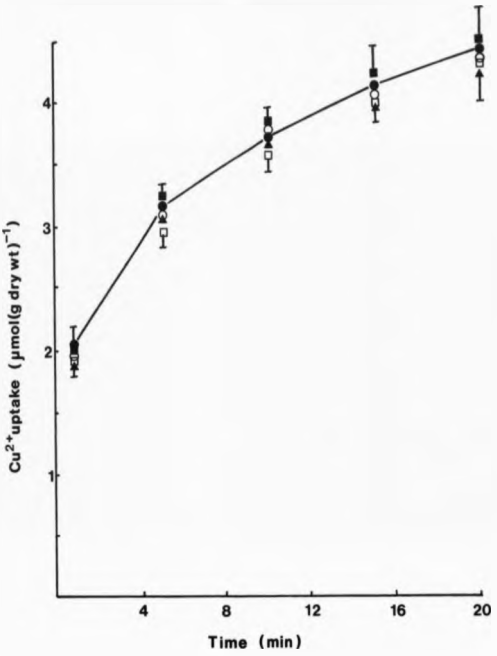


Figure 6.3.1. The uptake of Cu^{2+} from 5 µM solution in the absence (●) and in the presence of 50 µM additions of Mg^{2+} (○); Mn^{2+} (▲); Zn^{2+} (■) and Co^{2+} (□). Values represent the means ± SE of 3 experiments (for clarity only the maximum standard error bars have been included).

up to 20 min, by Mg^{2+}, Mn^{2+}, Zn^{2+} or Co^{2+}. The results are also given in tabulated form, for ease of comparison with Mn^{2+} uptake, in Table 6.3.1. The tabulated values have been corrected to account for surface-bound metal and so give a more accurate indication of the amount of metal which is actually internalised.

6.3.2. Effect of divalent cations on uptake from high and low manganese concentrations

Table 6.3.1. also summarises the effect of several bivalent cations on Mn^{2+} uptake from 50 μM Mn^{2+}. This data has also been corrected to account for surface-bound metal. The data for Mn^{2+} and Cu^{2+} may not be directly comparable, since the uptake was from differing concentrations due to the toxic nature of Cu^{2+} at higher levels. Nevertheless, a general effect can be seen; concentrations of other cations, twice that of Mn^{2+}, caused considerable inhibition of Mn^{2+} uptake in the decreasing order, Mg^{2+}, Co^{2+} and Zn^{2+}. However, Cu^{2+} uptake, in the presence of levels of Mn^{2+}, Mg^{2+}, Co^{2+} and Zn^{2+} 10-fold that of Cu^{2+}, was not significantly affected.

Table 6.3.2. shows the effect of bivalent cation competition on ^{54}Mn uptake from 10 nM ^{54}Mn. Due to the slight decrease in respiration which was previously observed following 30 min incubation in 10 μM Cu^{2+} solution (see Section 4.3.2.), the effect of 10 μM Cu^{2+} on ^{54}Mn uptake was not examined. Uptake was unaffected by 100-fold molar excess of other bivalent cations. In the presence of 1000-fold molar excess of Mg^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}, uptake was still 57 to 78 % of the original value, whilst 1000-fold excess Ca^{2+} exerted a somewhat less inhibitory effect, reducing uptake by only 2.3 %.

Table 6.3.1. Effect of divalent cation competition on Mn^{2+} and Cu^{2+} uptake

Competing metal ion	Mn^{2+} uptake % control	Cu^{2+} uptake % control
Zn^{2+}	81.1 (71.4 - 90.3)	107.2 (98.8 - 113.5)
Co^{2+}	44.5 (37.2 - 51.8)	95.8 (90.6 - 100.3)
Mg^{2+}	19.2 (15.5 - 23.2)	101.9 (93.2 - 109.3)
Mn^{2+}	-	100.1 (89.8 - 108.9)

Values shown represent the mean of 3 experiments (lower and upper limits of S.E. range in parentheses) and are for uptake after 10 min from 50 μM Mn^{2+} and 5 μM Cu^{2+} in the presence of 100 μM and 50 μM competing cation respectively. Control values for uptake in the absence of any competing cation were 1.91 ± 0.34 μmol g^{-1} dry wt and 3.74 ± 0.11 μmol g^{-1} dry wt for Mn^{2+} and Cu^{2+} respectively.

Table 6.3.2. Effect of divalent cation competition on ^{54}Mn uptake

Competing cation	^{54}Mn uptake (% control) in the presence of:	
	1 μM Competing cation (100-fold excess)	10 μm Competing cation (1000-fold excess)
Mg^{2+}	91.5 (87.9 - 94.5)	77.9 (60.9 - 92.6)
Zn^{2+}	102.0 (87.4 - 112.0)	57.4 (51.8 - 63.0)
Co^{2+}	113.4 (89.5 - 126.1)	72.4 (61.1 - 82.4)
Ni^{2+}	109.6 (94.5 - 121.1)	61.9 (58.4 - 65.5)
Ca^{2+}	102.6 (91.2 - 111.3)	97.7 (85.4 - 106.4)
Cu^{2+}	103.2 (99.3 - 106.2)	N.D.

Values shown represent the mean of 3 experiments (lower and upper limits of S.E. range in parentheses) and are for uptake after 20 min from 10 nM ^{54}Mn in the presence of 1 μM (100-fold molar excess) and 10 μM (1000-fold molar excess) competing cations. Control values for uptake in the absence of any competing cation is 5.16 ± 0.38 nmol ^{54}Mn (g dry wt)$^{-1}$. (N.D. = not determined)

6.3.3. Effect of copper on divalent cation uptake

During a preliminary investigation into the effect of other divalent cations on Mn^{2+} uptake from 50 μM Mn^{2+}, it was observed that Cu^{2+} appeared to exert a strong stimulatory effect on Mn^{2+} uptake whereas this effect was not seen during uptake from 10 nM ^{54}Mn (Table 6.3.2.). Figure 6.3.2. shows the effect of 1 μM Cu^{2+} on Mn^{2+} uptake from 10 μM Mn^{2+} in the presence and absence of DNP. In the presence of DNP, no Mn^{2+} uptake occurred, however in the absence of DNP, Cu^{2+} stimulated the rate of Mn^{2+} influx by a factor of 2.25. Cu^{2+} did not appear to affect initial Mn^{2+} surface binding.

To investigate whether this effect was limited to Mn^{2+} uptake, uptake of Zn^{2+} and Co^{2+} from 10 μM was also examined in the presence of 1 μM Cu^{2+}. Figures 6.3.3. and 6.3.4. describe the results. Cu^{2+} increased Zn^{2+} infux less dramatically than Mn^{2+} influx, stimulating the Zn^{2+} uptake rate by a factor of 1.32. Again, surface binding was not affected (Fig. 6.3.3.). The effect of Cu^{2+} on Co^{2+} uptake was somewhat different; the rate of uptake was unchanged yet the initial surface binding of Co^{2+} appeared to be halved and this initial decrease remained constant throughout the uptake period (Fig. 6.3.4.).

In order to further investigate the stimulatory effect of Cu^{2+} on Mn^{2+} uptake, the effect of both metals together on oxygen uptake was tested. Oxygen uptake rates were monitored after 6 min of incubation and remained linear for the following 10 min of measurement. Taking the uptake rate in the absence of any metal to be 100 %, the rate in the presence of 1 μM Cu^{2+} alone was 101.1% (range 99.3 - 102.7 %) whilst the rate in the presence of both 1 μM Cu^{2+} and 10 μM Mn^{2+} was 105.6 % (range 101.5 - 108.7 %) (values quoted are for the mean and

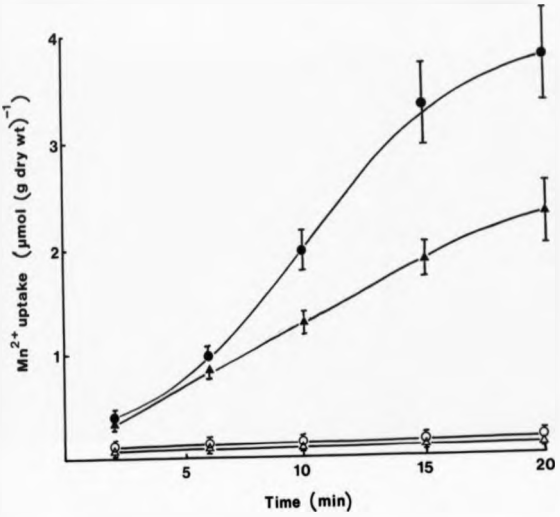


Figure 6.3.2. Mn^{2+} uptake from 10 µM Mn^{2+} in the absence (▲, △) and presence (●, ○) of 1 µM Cu^{2+}, and the absence (●, ▲) and presence (○, △) of 2 mM DNP. Values represent the mean ± SE of 3 experiments.

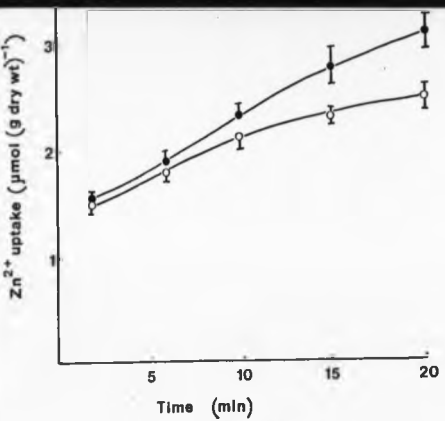


Figure 6.3.3. Zn^{2+} uptake from 10 µM Zn^{2+} in the absence (○) and presence (●) of 1 µM Cu^{2+}. Values represent the mean ± SE of 3 experiments.

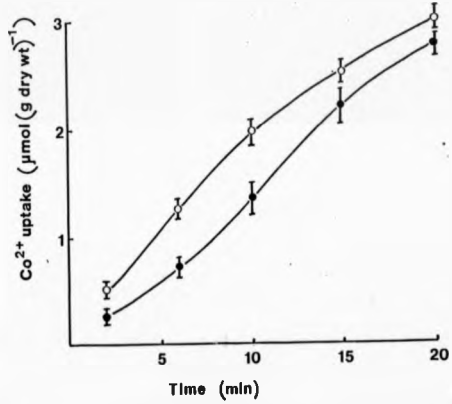


Figure 6.3.4. Co^{2+} uptake from 10 µM Co^{2+} in the absence (○) and presence (●) of 1 µM Cu^{2+}. Values represent the mean ± SE of 3 experiments.

S.E. range of 4 determinations). Thus, no significant increase in respiration was observed either in the presence of Cu^{2+} alone or Cu^{2+} and Mn^{2+} acting together.

6.4. Discussion

At high external concentrations of Mn^{2+} (50 μM), Mn^{2+} uptake was clearly inhibited very strongly by a 2-fold concentration of Mg^{2+} and, to a lesser extent, by Co^{2+} and Zn^{2+}, the inhibition series by these cations being, $Mg^{2+} > Co^{2+} > Zn^{2+}$. At the Mn^{2+} concentrations used, it appears that uptake does not occur *via* a specific system, and that Mn^{2+} is probably entering the cell through a general Mg^{2+} transporter, similar to that observed in *S.cerevisiae* (Borst-Pauwels, 1981). The fact that Mg^{2+} caused the greatest inhibition further strengthens the argument that Mg^{2+} is the primary substrate for this transporter and the lesser inhibitory cations (and Mn^{2+} itself) act as alternative substrates, although it cannot be determined from this limited study whether the metals all bind to the same transport site. These results are consistent with the affinity series seen in *S.cerevisiae* by several other authors (Rothstein, 1958, Putrament *et al*, 1977). Fuhrmann and Rothstein (1968) reported a greater affinity for Zn^{2+} than for Mn^{2+} whilst the data presented here clearly shows a relatively high Mn^{2+} uptake. This may simply be due to a yeast species difference, however to obtain an accurate picture of the actual affinities and the nature of transport inhibition, a kinetic analysis of this transport system is required. It is worthy of mention here of the reasons why Mg^{2+} was not used as the metal ion under investigation when the putative primary Mg^{2+} transporter was being studied. This was the result of two factors: yeast cells

generally contain high concentrations of intracellular Mg^{2+} making attempts to measure small Mg^{2+} fluxes into cells virtually impossible without the aid of the radioactive form of the metal. ^{28}Mg is of such short half-life and is of such limited availability that its use is rendered impractical, hence most studies of the Mg^{2+} carrier, including the present one, have examined it indirectly by the use of an alternative substrate, often Mn^{2+}.

When the external media contains very low Mn^{2+} concentrations (10 nM), ^{54}Mn uptake appears to be *via* a highly specific mechanism, unlike the low affinity cation transporter previously described. The degree of cation specificity is not as great as that observed by Silver *et al* (1970) for Mn^{2+} transport in *E.coli*, which was largely unaffected by 10^6-fold molar excess of Mg^{2+}, Ca^{2+} or Sr^{2+}. Nevertheless the present yeast system exhibits considerable discrimination for Mn^{2+} in the presence of 1000-fold excess of Mg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} or Ca^{2+}. Specific Mn^{2+} uptake in *L.plantarum* (Archibald & Duong, 1984) and in *B.subtilis* (Eisenstadt *et al*, 1973) was unaffected by 100-fold molar excess of Cu^{2+}, Fe^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+} or Ca^{2+} and by 10^5-fold excess of Mg^{2+} and Ca^{2+} respectively. More relevent to the present study, the Zn^{2+} transporter of *C.utilis* shows a similar degree of specificity with 100-fold excess of Na^+, Ca^{2+}, Cr^{3+}, Mn^{2+}, Co^{2+} or Cu^{2+} having no effect on uptake (Failla *et al*, 1976).

A number of previous reports have detailed the mild inhibition of specific transporters by several cations. Zn^{2+}-specific uptake in *C.utilis* was inhibited by Cd^{2+} (Failla & Weinberg, 1977) whilst Cd^{2+} is also reported to share the specific Mn^{2+} carrier in a number of bacteria (Archibald & Duong, 1984, Laddaga *et al*, 1985, Trevors *et*

al, 1985). Cd^{2+} was not included in this study as yeasts have no essential requirement for this ion and a number of deleterious effects of Cd^{2+} on cells have been reported (Grafl & Schwantes, 1983a, Gadd & Mowll, 1984, Jones & Greenfield, 1984). Eisenstadt *et al* (1973) also showed that Co^{2+} and Fe^{2+} acted as very low affinity inhibitors of specific Mn^{2+} influx. It is not unreasonable to expect that there will be occasional overlap between structurally or functionally related cations, but it is unlikely that these specific mechanisms would be influenced by the major cations, such as Mg^{2+} or Ca^{2+}, normally present in the cell's environment.

Thus, in *C.utilis*, under usual growth conditions (ie. low Mn^{2+} concentrations), the highly specific Mn^{2+} system will most likely predominate and provide much of the cellular Mn^{2+}. At high external Mn^{2+} however, Mn^{2+} enters the cell by the putative Mg^{2+} transport system. In *E.coli*, Silver (1978) has calculated the threshold concentration, below which the specific Mn^{2+} carrier predominates and above which the Mg^{2+} carrier is the principal source of Mn^{2+} transport, to be around 40 μM Mn^{2+}.

The interaction of other bivalent cations with Cu^{2+} uptake was of interest, particularly since, except for a recent study by Gadd and Mowll (1985) in the dimorphic fungus *A.pullulans* which prevails in metal-polluted environments, there is little reliable information regarding the specificity of uptake of this essential metal ion. This present study has shown that Cu^{2+} uptake was unaffected by a number of other common divalent cations, present at 10 times the Cu^{2+} concentration. This data, together with the reduced sensitivity of Cu^{2+} uptake to certain inhibitors (see Chapter 5) as compared to Mn^{2+} uptake through both the high and low specificity systems, strongly

suggests that Cu^{2+} transport is *via* a separate and more specific system. The range of competing cations used in this study was not comprehensive, yet the primary substrates of the demonstrated and putative divalent cation transport systems in *C.utilis* were all tested to no effect on Cu^{2+} uptake. Whilst the possibility of other cations inhibiting this system cannot be precluded, in view of the demonstrated essentiality of Cu^{2+} in *C.utilis* (Light, 1972, Downie & Garland, 1973) and on the basis of the present results, it is possible that a specific Cu^{2+} transport system might exist to provide the cell with sufficient Cu^{2+}. As there are no directly comparable Cu^{2+} uptake studies, either in fungal or bacterial species, it is difficult to assess the importance of such a system and whether it is peculiar to *C.utilis*. It would be interesting to apply a similar study to the glucose-sensitive yeast, *S.cerevisiae*.

In view of the high degree of specificity exhibited by uptake systems for metal ions, the question arises: how do cells discriminate so efficiently for a particular cation? Clearly, the uptake of these elements and their use in the cell depends upon the chemical and physical properties of each element. In two useful reviews on the physico-chemical aspects of ion selectivity and transfer through biological membranes, Diamond and White (1969) and Williams (1981) determined a number of parameters which govern the uptake of metal ions in living cells and these have been summarised by Wood (1984a) as follows:

1. Charge
2. Ionic radius
3. Preference for the coordination of metals to certain organic ligands

4. Coordination geometry and coordination numbers for retention
5. Spin pairing between metal ions for more stability and the degree of covalence of metal-ligand interactions
6. Available concentrations of metal ions in the aqueous environment
7. Kinetic controls which are pertinent to metal ion transport and binding
8. The chemical reactivities of metal ions in solution

Thus, it can be seen that the selectivity of a membrane site for a metal ion is not straightforward and that complex interactions may give rise to a transport system which is specific for a particular ion.

A number of authors have, not unreasonably, placed the emphasis on crystal ionic radii of metals as a basis of their selection. In order of decreasing size, the ionic radii of divalent cations is (values, in nm, given in parentheses): Pb^{2+} (0.120), Sr^{2+} (0.112), Ca^{2+} (0.099), Cd^{2+} (0.097), Mn^{2+} (0.080), Zn^{2+} (0.074), Fe^{2+} (0.074), Co^{2+} (0.072), Cu^{2+} (0.072), Ni^{2+} (0.069) and Mg^{2+} (0.066) (CRC Handbook of Chemistry & Physics, 1974). Some correlation of this series with the degree of inhibition of uptake of Ca^{2+}, Cd^{2+} and Co^{2+} in yeasts has been noted (Borst-Pauwels, 1981) and it was observed that the inhibition was approximately maximal when the difference in ionic radius between the substrate cation and inhibitory cation was minimal. In addition, cations require a similar charge, or valency state, before the criterion of ionic radius can determine competition for uptake (Norris & Kelly, 1977). The similarity in ionic radii may well explain the strong inhibition of Cd^{2+} uptake by Ca^{2+} (Mowll & Gadd, 1984) as the ionic radius differs by only 0.002 nm. It is possible that, as Ca^{2+} has a larger ionic radius than the other cations tested for inhibition of specific ^{54}Mn uptake at 1000-fold

molar excess, it had the smallest inhibitory effect.

Whilst there are several clear examples of the relationship between inhibition and ionic radius, there are an equal number of studies in which the degree of inhibition bears no resemblance to ionic size. This is also the case for the highly specific systems which have been observed. In this study, divalent cation inhibition of Mn^{2+} uptake *via* both the high and low specificity systems could not be correlated to crystal ionic radii of the cations used. Indeed, the inhibition of Mn^{2+} uptake by certain metals may be difficult to explain. The preference of the Mn^{2+} carrier for Cd^{2+} in *L.plantarum* was unusual in that Mn^{2+} shows greater similarity to transition metals, such as Co^{2+} or Fe^{2+}, in terms of magnetic properties, mass, crystal and hydrated radii and electron shell configuration (Archibald & Duong, 1984). The authors suggested that, as they could demonstrate that hexaquo Mn^{2+} ions did not appear to be as available to cells as Mn^{2+} which was complexed or distorted by any of a number of anions, then the degree of hydration and electron cloud shape of the Mn^{2+} recognised by the Mn^{2+} uptake system may be very different to those of free Mn^{2+}. The order of stability of complexes formed with organic ligands *viz.*:
Cu > Ni > Pb > Co > Zn > Cd > Mn > Mg > Ca (Bowen, 1966) may play a role in determining the selectivity of a transport system as might the similar order of solubility products of the metal salts, although again there is no one series of cation affinities for a transporter which can be exactly related to this data. It seems that it is probably a combination of physico-chemical parameters which ultimately determines cation selectivity.

The availability of different metal ions to *C.utilis* cells in this study would most likely be little affected by the MES buffer used. Metal-binding constants of MES are not available for all the metals studied, however the values for Mn^{2+}, Mg^{2+} and Ca^{2+} are all listed as similar (Good *et al*, 1966), thus the apparent specificities of cells for metal ions being due to the differential availability of the metal in solution is most unlikely. Furthermore, at the large molar excess at which the competing cations were added, the specificities of Mn^{2+} and Cu^{2+} uptake are most obvious.

It is probable that the metal binds to a complex transport site to achieve this level of selectivity and there exist a number of possible binding molecules. Pyridoxial, polyphosphates and phosphoryl choline have been suggested by several authors as possible cation transport carrier molecules (Jennings *et al*, 1958) and the Cu^{2+} transport process in *Debaryomyces hansenii* involves the participation of a carrier and a thiol compound (Wakatsuki *et al*, 1985). Non-proteinaceous ionophores can discriminate greatly between ions. The ionophore, A23187, acts as a specific porter of divalent cations, having a greater affinity for Mg^{2+} than for Ca^{2+} (Jasper & Silver, 1977) and has been previously applied to yeast studies (Duffus & Patterson, 1974). Failla *et al* (1976) suggested that 'de novo' protein synthesis was required for Zn^{2+} transport in *C.utilis*. Foradori *et al* (1967) clearly demonstrated the ability of proteins to markedly discriminate between Mg^{2+} and Mn^{2+} and it seems quite possible that some protein involvement may contribute to the specificity observed in this study. It is predicted that bacterial cation transport systems will consist of membrane embedded carriers, undoubtedly proteins, determined by genes on the bacterial chromosome and with expected recognition sites hypothetically determined by

amino acid sequences within the carrier membrane proteins (Silver, 1978, 1983). Using agents such as formaldehyde to 'seal' the cell, Archibald and Duong (1984) suggested that Mn^{2+} enters *L.plantarum via* proteins and not *via* the lipid bilayer of the membrane while Rothstein (1958) indicated that the specificity pattern of Mn^{2+} transport in *S.cerevisiae* strongly implied a close association with a protein, in a similar manner to phosphorylation enzymes with Mg^{2+} requirements, rather than binding to fixed ionic groups, such as phosphoryl or carboxyl (Rothstein *et al*, 1958). It was suggested that the carrier system involved a phosphorylated substance and a protein constituent, both acting together to confer the observed specificity. Taking known Ca^{2+} translocating ATPases, a molecular model for the mechanism of transport has been postulated involving a conformational change in a membrane-spanning molecule (Scarborough, 1985) and Kotyk and Janacek (1975) deduced that, as no other type of substance was known to possess such selective qualities, the binding sites of transport systems must be of protein character. However, they also pointed out that specificity may be determined, not by the mobile membrane component (if the carrier exists in this form), but by an enzyme catalysing substrate binding to the carrier and containing binding loci for both. They reported that some evidence for this has been seen in erythrocyte sugar transport.

There is little data available on the mechanism whereby one metal increases the uptake of a second metal. In this study, the rate of Mn^{2+} uptake and, to a lesser extent, Zn^{2+} uptake was increased by low concentrations of Cu^{2+}. Several previous reports (see, for example, Gadd & Mowll, 1985) have shown that metals which are known to have a disruptive action on cell membranes can increase intracellular absorption in yeasts through passive influx due to the increased

permeability of the membrane. This could be ruled out in this case, as the increased Mn^{2+} influx in the presence of Cu^{2+} was also dependent on cellular metabolism, being completely suppressed in the presence of the metabolic poison, DNP. In *S.cerevisiae*, increased medium Cu^{2+} led to increased uptake of Zn^{2+} though not Mn^{2+}, whilst at optimal Cu^{2+} levels Mn^{2+} uptake is elevated 2-fold above that for suboptimal Cu^{2+} (Gesswagner & Altmann, 1976). This effect was attributed to an increase in -SH and amino groups formed in the yeast cell for which Cu^{2+} and Zn^{2+}, but not Mn^{2+}, have an affinity. This explanation does not sufficiently account for the present results, as the stimulatory effect was observed immediately in cells which had been grown and preincubated under identical conditions. Increased accumulation of both Mn^{2+} and Zn^{2+} in the presence of Cu^{2+} has also been observed in growing cultures of *C.utilis* (Ross, unpublished observations).

The activation of respiration by metal ions (Carafoli *et al*, 1970) and the stimulation of glycolytic flux in *S.cerevisiae* by 1 μM Cu^{2+} (Jones & Greenfield, 1984) has been observed, however no significant increase in oxygen uptake could be detected here when Cu^{2+} was added either alone or in combination with Mn^{2+}. As *C.utilis* is a highly respiratory yeast, it seems unlikely that any major stimulation of metabolism occurred leading to accentuated Mn^{2+} uptake, particularly as the Mn^{2+} uptake rate was increased by well over 2-fold.

In the few reports mentioning synergism of metal uptake by other cations, little attention has been paid to this phenomenon. Initial uptake of Mn^{2+} and Ni^{2+} in *S.cerevisiae* was increased 7-fold and 2-fold respectively by Cu^{2+} (Norris & Kelly, 1979) whilst Zn^{2+} uptake in *C.utilis* was doubled by Ni^{2+} and Fe^{2+} (Failla *et al*, 1976), and

Zn^{2+} uptake in *S.cerevisiae* was increased by Co^{2+} and Co^{2+} uptake increased by Zn^{2+} (Fuhrmann & Rothstein, 1968). In bacterial studies, Mn^{2+} could act in a synergistic or antagonistic manner on Mg^{2+} uptake according to its initial concentration (Webb, 1969), Silver *et al* (1970) observed the stimulation of Mn^{2+} transport by Cu^{2+} in *E.coli*, and Ca^{2+} transport in *B.subtilis* membrane vesicles was strongly increased by Mg^{2+} (de Vrij *et al*, 1985). A number of these above reports have attempted to explain this effect by proposing that the stimulatory cation may displace surface-bound metal from sites on the cell wall, thus increasing the effective concentration of the metal available for uptake. Certainly, Cu^{2+} is known to have a high affinity for wall sites; in *A.pullulans* its binding is unaffected by other metals (Gadd & Mowll, 1985), however, in all the experiments used in this study the total metal concentration of the buffer, before and after the uptake period, changed very little and the amount of metal which might be displaced from non-specific binding sites would be very low compared with the effective external metal level. Indeed, in the case of Co^{2+} uptake, Cu^{2+} may have displaced the surface-bound Co^{2+} initially but had no effect on the subsequent Co^{2+} internalisation rate. By examining the altered uptake profiles, it can be concluded that Cu^{2+} did not affect surface-bound Mn^{2+} or Zn^{2+} as only the prolonged uptake rate and not the initial binding was increased. It is difficult to explain why this selective stimulation occurs. It is not compatible with a single site uptake mechanism (Borst-Pauwels, 1981) and indicates that the stimulatory cation is not a substrate for the transport system investigated (de Vrij *et al*, 1985). Neither is it known whether this is due to a direct effect or an indirect effect *via* the membrane components on the carrier (de Vrij *et al*, 1985). It is possible that Cu^{2+} acts at the level of the uptake system and interferes with the

control of Mn^{2+} and Zn^{2+} transport in some way or alternatively affects the cell membrane potential although these effects are not obvious. It should be noted that no stimulatory action of Cu^{2+} was observed during Mn^{2+} uptake from low Mn^{2+} levels by the putative Mn^{2+}-specific uptake system, further evidence that two systems do exist which are capable of transporting Mn^{2+}. Clearly, interactions between trace elements with respect to metal uptake in yeasts require further investigation.

In conclusion, this section of the study was hampered by the lack of available data on specific metal transporters in yeast and fungal cells, with the only corresponding studies of this type being in bacterial species. However, it has been clearly demonstrated that all bivalent metal ions do not share one transport mechanism. Indeed, the data indicates that at least three separate transporters; a non-specific system (possibly the primary Mg^{2+} carrier) and specific Mn^{2+} and Cu^{2+} carriers, are involved in divalent cation translocation, with the additional data of Failla *et al* (1976) suggesting that a fourth system, the Zn^{2+} carrier, may operate in *C.utilis*. So far, five specific cation transporters have been observed in the bacteria, *B.subtilis* and *E.coli* (Eisenstadt *et al*, 1973), and these specific micronutrient carriers are perhaps analogous in specificity and multiplicity to amino acid transport systems (e.g. Anraku, 1978). The next section of this study will attempt to define the kinetic parameters of each of these transport mechanisms and to give an accurate indication of the relative affinities of the substrate and inhibitor cations for these systems.

7. KINETIC ANALYSIS OF TRANSPORT

7.1. Introduction

In any uptake study, one of the primary aims is to establish the kinetic parameters of the uptake process. Once the cellular uptake of a solute has been demonstrated, then by examining the effect of varying solute concentrations on uptake rate, the uptake system can be characterised and quantified in terms of its kinetics. In addition, the nature of inhibition of a system by analogous solutes or other inhibitors may also be determined. Hence, by obtaining values for the affinities of the transport systems for the substrates and values for the maximal transport rates, each separate system can be accurately characterised. This data can then be used to differentiate between micro- and macro-nutrient transport systems, as the kinetic parameters of various ion transport systems frequently reflect the intracellular concentrations of each ion required by the cells (Jasper & Silver, 1977). It should be noted that of the numerous studies of metal uptake in yeasts, a great many have used complex incubation media and differed in experimental design, yielding non-comparable kinetic data. In the last chapter, the presence of three putative transport systems was demonstrated; two highly specific systems for the uptake of Mn^{2+} and Cu^{2+} respectively and a general divalent cation system of low specificity. A comprehensive study of the uptake rates of these essential metals under standardised conditions is needed to give directly comparable and quantitative information as to their affinities for cations and to confirm the multiplicity of the transport systems outlined in the previous chapter.

7.2. Theoretical background

7.2.1. Determination of the transport constant and the maximal rate of transport

Whether ion transport occurs *via* specific ion 'pumps' or is mediated by carrier molecules (both systems having a finite number of transport sites) it is a characteristic feature of these mechanisms that they can become saturated with the transported solute. As a result of this, uptake is not linear with respect to the solute concentration. The processes of ion binding to the transport site, transport across the membrane and release inside the cell all serve to limit the rate of transport. A striking similarity can be seen between this and the binding of a substrate to an enzyme and the subsequent breakdown of this complex and release of product. Indeed, the application of kinetic analysis to transport data rests on the assumption that the membrane transport sites of the cell are analogous to the active reaction sites of an enzyme and that transport of the solute into the cell is equivalent to the formation of the enzyme product.

From this analogy, an expression has been derived for the mathematical treatment of carrier-mediated transport (Neame & Richards, 1972, Zivin & Waud, 1982) and is shown below:

$$v = \frac{V_{max} \cdot [S]}{[S] + K_t}$$

v is the rate of uptake at a solute concentration S. V_{max} is the maximum uptake rate at saturating conditions and can also be taken as

an indirect measure of the number of transport sites per unit biomass. K_t, the transport or saturation constant, is numerically equal to the concentration of solute at half maximal uptake and is an indication of the affinity of the transport site for the solute. The lower the value of K_t, the greater the affinity. This relationship, known as the Michaelis-Menten equation, was originally devised to describe enzyme kinetics, the difference being that the Michaelis constant, K_m, is a measure of the enzyme's affinity for the substrate. Hence much of the terminology used in enzyme kinetic analysis is similar to that used in transport studies.

There are important differences between the two systems and a number of assumptions are made. The Michaelis-Menten equation depends upon unidirectional transport, that is, it does not account for solute efflux. Therefore the assumption is made that net uptake is equivalent to influx. In practise however, it is difficult to accurately separate influx from efflux and so transport is measured over a short time interval during which the uptake of solute is linear with respect to time. Hence initial-rate transport is presumed to be an indicator of the unidirectional influx of a solute, this being based on the assumption that the amount of intracellular solute accumulated is small enough for outward transfer to be ignored (Neame & Richards, 1972). Certainly this is likely to be the case for metal ion transport where it is generally thought that a large proportion of cellular metal is bound to cell constituents and is not freely available in the cytoplasm. As long as the initial rate of uptake of a solute is linear, the Michaelis-Menten equation may be accurately applied to analyse the transport kinetics and so preliminary studies of uptake with time must be carried out to ensure no deviations from linearity occur. It is also assumed that the

effective solute concentration is not significantly reduced during uptake.

Once the initial period of uptake has been determined, many investigators use this fixed time interval over which to measure uptake and, during all subsequent experiments, assume linearity of uptake over this period. In this study, due to small variations in apparent uptake resulting from experimental error, it was considered a more reliable method to fully plot solute uptake at all time intervals for each solute concentration used, thus ensuring linearity of uptake.

When the initial rate of uptake, v, is plotted against substrate concentration, S, a rectangular hyperbola which passes through the origin is obtained (Fig. 7.2.1.). The kinetic parameters of transport, K_t and V_{max}, can be determined directly from this plot, however it is not easy to construct a hyperbola through a set of experimental points, particularly if the measurements do not extend to the plateau portion of the curve. Without an accurate extrapolated value for V_{max}, the 1/2 V_{max} and hence K_t cannot be determined. In order to circumvent this problem, the Michaelis-Menten equation can be transformed in several ways to give a linear plot. Of the various forms, the Lineweaver-Burk plot has become the most well-known (Lineweaver & Burk, 1934). Taking reciprocals of both sides of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{K_t}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

This is of the form $y = mx + c$ (the equation for a straight line) and so when 1/v is plotted against 1/[S], the resultant plot is linear with a slope of K_t/V_{max} and the ordinate intercept equal to $1/V_{max}$ (Fig. 7.2.2.). There are also two other linear arrangements of the Michaelis-Menten equation, namely plots of [S]/v against [S] (Woolf, 1932) and v against v/[S] (Hofstee, 1956). Several other statistical methods have been applied to extract data for K_t and V_{max} and these include a computer curve fitting exercise to fit values of v and [S] to the rectangular hyperbola (Cleland, 1967) and the direct linear plot as described by Eisenthal and Cornish-Bowden (1974) where each measurement is represented by a line rather than a point.

There appears to be advantages and disadvantages in using all of the aforementioned methods and these have been critically evaluated by Markus *et al* (1976). The most extensively used transformation is the Lineweaver-Burk (or double reciprocal) plot. The main argument against the use of this plot is that it biases the interpretation of experimental results and gives undue weight to inaccurate measurements at low solute concentrations and insufficient weight to the more accurate higher rates (Engel, 1977). Consequently there is a heterogeneity of variance at all places along the line, this being much greater at low solute concentrations. It does have several advantages however, the independent variables, v and [S], are kept separate and are easy to comprehend whereas the other plots involve compound variables. It is also simple to plot and is well-known.

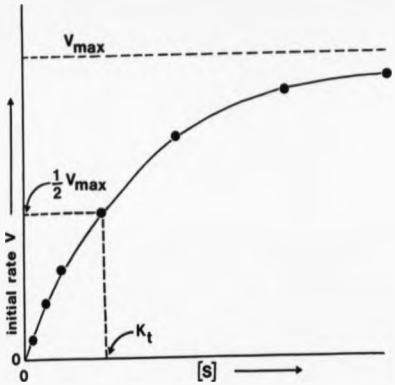


Figure 7.2.1. Dependence of initial transport rate on solute concentration for a typical carrier-mediated transport process.

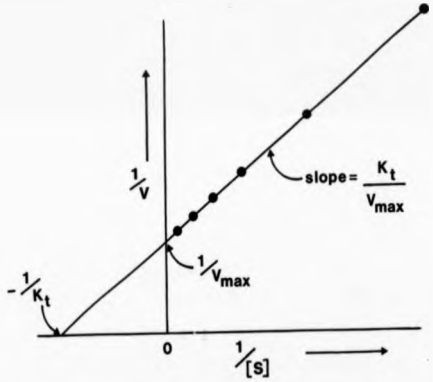


Figure 7.2.2. A Lineweaver-Burk plot

Partly due to its familiarity but in the main for reasons of comparison, as Lineweaver-Burk plots have been almost exclusively used in studies of metal uptake by microorganisms, this was the plot of choice in the present study. With the criticisms in mind it is worth mentioning that, due to the sensitive analytical techniques developed, the determinations of uptake at the lowest solute concentrations investigated were well within the detection limits of the method used and thus the variability of these measurements was low. In addition, Dowd and Riggs (1965) showed that if the low rate determinations were weighted, the Lineweaver-Burk plot had the same degree of accuracy as the Woolf plot which is free from the above criticism. Hence, when a plotted locus in this region appeared abnormally small or large and clearly did not fit the linear trend of the other data, it was generally ignored in this study. As the main criticism against this plot concerns the bias of low rate measurements, it is believed that these factors would minimise this bias and produce reasonably accurate determinations of the apparent K_t and V_{max} of the yeast cell transport systems.

7.2.2. Determination of the inhibitor constant

The rate of transport may sometimes be altered in a specific manner by substances other than the solute being measured. Analogues of the test solute can inhibit the transport rate and may act as alternative substrates for the transport system. The degree and type of inhibition can be examined by varying the concentration of the solute, S, whilst maintaining a fixed concentration of the inhibitor, I. The type of inhibition can be examined when data of the uptake in the presence and absence of the inhibitor is plotted by the Linneweaver-Burk method as before. Competitive inhibition, where the test solute and inhibitor both compete for the same transport site,

results in a higher level of S required in order to approach the V_{max} and accordingly the K_t is raised (Fig 7.2.3.). The V_{max} does not vary in this instance.

Non-competitive inhibition is shown in Figure 7.2.4. Here, the inhibition is due to effects of the inhibitor other than at the solute binding site of the transport system. An example of this might be the effect of an inhibitor at a site of action on the metabolic machinery of the cell. This results in a reduction of the V_{max} but has no effect on the apparent affinity of the transport site.

The inhibitor constant, K_i, is the concentration of the inhibitory analogue which is sufficient to reduce the transport rate to half its maximum (Christensen, 1975) and is a useful measure of the potency of various inhibitors. The constant can be mathematically quantified by substitution into the equation:

$$\text{INHIBITED SLOPE} = (\text{UNINHIBITED SLOPE})(1 + [I] / K_i)$$

where the gradient of the uninhibited slope is equal to K_t/V_{max} as before. Thus, the K_i can give an indication of the affinity of the transport site for the inhibitor and is in many ways similar to K_t. If the competitive analogue can also serve as a substrate, as is often the case in transport studies, with the same affinity for the transport site as the test solute, then the values of K_t and K_i should be equal.

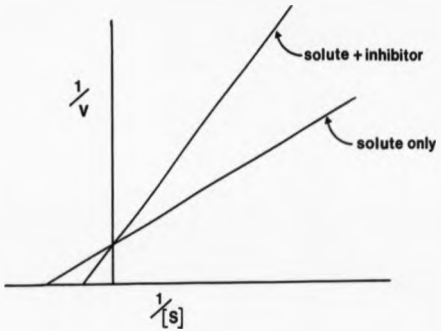


Figure 7.2.3. Competitive inhibition as seen in the Lineweaver-Burk plot

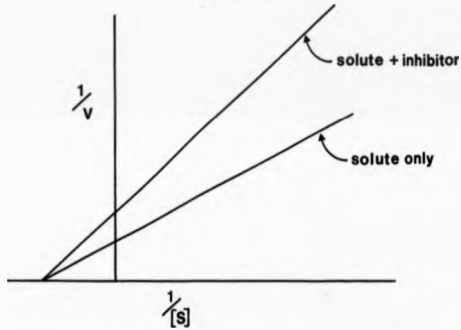


Figure 7.2.4. Non-competitive inhibition as seen in the Lineweaver-Burk plot

7.3. Statistical analysis of kinetic data

The predicted data obtained from transport kinetic studies is subject to varying degrees of experimental error and, whilst the graphical analysis is a simple procedure, it does not provide estimates of the reliability of the constants obtained. To fit a line to the experimental loci on a Lineweaver-Burk plot, the method of least-squares regression analysis can be applied as described by Cleland (1967). From this, the intercept on the *y*-axis and the slope of the line can be determined. It is equally important to obtain some index of the precision of the estimates of K_t and V_{max}, so that their significance can be assessed. The degree of uncertainty in these estimates can be quantified by calculating the standard error of estimate (S.E.E.) for each data set from the formula:

$$S_{x(\text{or } y)} = \sigma_{x(\text{or } y)} \cdot (1 - r^2)^{1/2}$$

where σ = the standard deviate of the particular data and r = the product moment correlation coefficient. The variation in the K_t and V_{max} values can be calculated from the *y*-intercept values $\pm$ S.E.E. In 95 % of cases, the actual values fall within $\pm$ 2 x S.E.E. of the estimate values (Moroney, 1978). Thus data for K_t and V_{max} can be provided with maximum and minimum values which are 95 % confidence limits. This type of analysis, giving a range for each value, has been previously used to interpret transport data (eg. Jeffs & Arme, 1985) and, following a least-squares fitting exercise, was the method of choice in this study.

7.4. Factors affecting uptake kinetics

7.4.1. Effect of surface potential

The outer surface of the yeast cell membrane has a series of fixed negative groups which give rise to a net negative charge or negative surface potential at the membrane surface. The presence of this negative potential in the region of the binding sites for cation transport will lead to an elevated accumulation of cations and an exclusion of anions in that region, thus influencing the kinetics of cation transport (Borst-Pauwels, 1981). On the addition of salts, such as $MgCl_2$ or KCl, the surface potential will decrease, becoming less negative, and this is mainly due to the additional cations screening the surface negative charges. The screening ability of cations greatly increases with the valency state of the cation rather than the ionic strength (Mills & Barber, 1978) so that the efficacy of a cation, C, at reducing the surface potential decreases in the order, $C^{+++} > C^{++} > C^{+}$. In addition, some binding of cations to fixed negative groups occurs which may contribute to this effect (McLaughlin *et al*, 1971).

The effect on kinetics has been largely ignored in most previous yeast transport studies, presumably due to the complexities involved in examining these interactions. However, recent data from Borst-Pauwels' laboratory has shown that, depending on the experimental uptake conditions, the inhibition of Rb^+ uptake caused by the reduction of the surface potential by polyvalent cations could be misinterpreted as being a competitive effect, as the K_t for Rb^+ transport was increased (Theuvenet & Borst-Pauwels, 1976). Furthermore, they also showed that Ca^{2+} diffusion into cells behaved kinetically distinct as being mediated by a two carrier system when

this could be ascribed to a reduction in surface potential concomitant with increasing Ca^{2+} concentrations (Borst-Pauwels & Theuvenet, 1984). Thus increasing the salts will have the effect of reducing the apparent affinity of the cation for the transport site (as the potential is less negative) (Borst-Pauwels, 1981). In practice, this type of effect has also been described by Roomans *et al* (1979) in *S.cerevisiae*. Ca^{2+} and Sr^{2+} transport in this species deviated from Michaelis-Menten kinetics at higher concentrations of the divalent cations and the uptake increased with decreasing bulk H^{+} concentrations coupled with a progressive increase in the surface potential.

To account for the possibility that changes in the surface potential might affect the apparent transport kinetics, a study was undertaken to attempt to estimate the surface charge density, and hence surface potential, of cells of *C.utilis* using the monovalent cationic dye 9-aminoacridine as a probe. This work has been included in Appendix I as, whilst it does not form part of the main body of this study, it nevertheless gives a useful indication as to the magnitude of the surface negative charges and enables some predictions to be made of the likely effects of the surface potential under the differing cation concentrations used in this chapter.

A surface charge density for *C.utilis* cells of approximately - 12 $\mu C\ cm^{-2}$ was estimated from this study. By applying the surface charge density to an equation which has been derived from the Gouy-Chapman expression by Barber *et al* (1977), curves describing the theoretical surface potential at varying mixed concentrations of mono- and divalent salts can be generated. Assuming a surface charge density of - 10 $\mu C\ cm^{-2}$, these curves have been computer-generated in

Figure 7.4.1. (although this was not the exact charge density estimated in Appendix I, it is a close enough approximation for the purposes of the present study). It can be seen that at a given monovalent cation concentration, increasing the divalent cation concentration has no effect on the magnitude of the surface potential until a threshold divalent cation concentration is reached, after which the surface potential decreases with increasing divalent cation concentrations. This threshold concentration is increased in the presence of higher monovalent salt concentrations. The monovalent salt concentrations selected for these curves was not fortuitous as all uptake experiments in this study buffered by MES were carried out in 10 mM KOH.

Hence it can be seen from Figure 7.4.1. that under the experimental conditions imposed throughout this study, the surface potential would not be significantly reduced until the divalent cation concentration exceeded 0.1 mM. Excepting studies of Mn^{2+} uptake from 0.1 mM to 1.0 mM Mn^{2+}, all uptake kinetic experiments in this chapter were from divalent cation concentrations well below this threshold. Therefore it can be predicted, on a theoretical basis, that any effects of the surface potential will be kept constant and that the kinetic measurements obtained should be free from interference by a decreasing surface potential.

As the situation in whole cells is somewhat more complex than in simple membrane systems, theoretical curves of this type should be used to predict effects on transport kinetics with some circumspection. However, knowing the predicted effect on kinetics, the theoretical data can be compared with observed kinetic data to determine whether or not these effects are likely to be significant

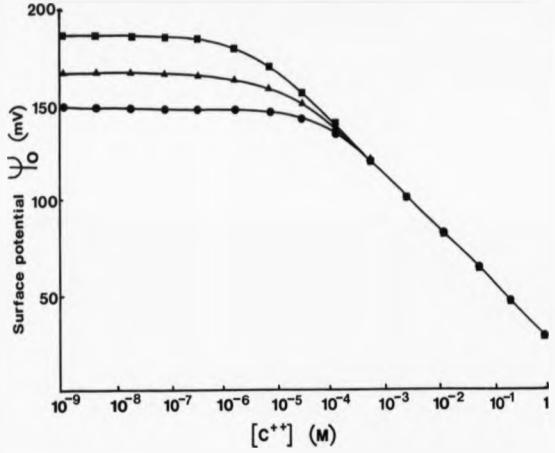


Figure 7.4.1. Computer-generated curves showing changes of surface potential for different levels of mixed electrolytes

The effect of changing divalent salts (C^{++}) (horizontal axis) is shown for different levels of monovalent salts (C^{+}), [(●) 10 mM C^{+}; (▲) 5 mM C^{+}; (■) 2.5 mM C^{+}].

in practice.

7.4.2. Effect of unstirred layers

As much of the kinetic considerations of membrane transport assume that the solute concentration immediately adjacent to the membrane is the same as that in the bulk medium, the situation can be further complicated by the presence of unstirred aqueous layers which can act as diffusion films. If the diffusion film is of considerable thickness then the concentration of solute at the surface of cells may be small compared to the bulk concentration, as transport of solute into the cells provides a continuous sink (Button,1985). Thus under certain circumstances, the rate-limiting step for solute transport could be the diffusion of the solute through an unstirred layer. Winne (1973) has demonstrated that unstirred water layers can give rise to an overestimation of the transport constant and thus a downward bias of the apparent affinity. This bias depends upon the thickness of the unstirred layer and gives rise to non-linear deformations of Lineweaver-Burk plots. By increasing agitation rates of the bulk medium, the effect of unstirred layers can be reduced (Gains, 1980).

Whilst this effect can be most pronounced in studies of transport in larger structures such as intestinal sacs, the effect on microbial transport is debatable. Yeast cells are enclosed in a rigid wall matrix which can account for 10 % of the total cell volume (Conway & Duggan,1950) and it is within this outer region that unstirred layers may be present. Since all uptake experiments were carried out using a constant shaking speed of 200 cycles min^{-1}, it is unlikely that diffusion shells would exist outside the cell wall region and the question is therefore, what effect the cell wall has on diffusion of

ions to the membrane transport sites. This remains unclear although there are indications that concentrations at the surface of bacteria in all but very fast growing systems are nearly as large as those in bulk solution (Button, 1985). In addition, Button states that of the numerous treatments of diffusive limitation, none clearly define the surface concentration and the gradients involved. Whilst the effect of the unstirred layer in yeasts may be small, it is an important point to bear in mind that the apparent solute concentrations in the transport kinetic equation can be different to the effective concentration as experienced by the transport systems in the membrane.

7.5. Materials and methods

The preliminary procedures used to prepare cell suspensions and uptake experiments are as previously described in Chapter 3. For competition experiments, competing metal chloride salts were added 15 s prior to the addition of the metal under investigation. To ensure rapid filtering and washing of cell samples, the sample volume removed was reduced to 15 ml in the case of non-radioactive experiments. In general, the uptake kinetics were determined from the linear rates of uptake during the initial 10 min of incubation with the metal. All experiments in this section were repeated at least 3 times using a different batch of cells on each occasion. Hence in all kinetic treatments of transport, the mean data of at least 3 separate experiments has been used.

All statistical analyses to determine the kinetic parameters and quantify their degree of uncertainty were performed using a

statistical software package on a GEC 4000 machine (University of Keele, Computer Centre).

7.6. Results

7.6.1. Kinetics of uptake from high manganese concentrations

As previously mentioned, the loci on Lineweaver-Burk plots were determined from the average rate of linear uptake of a number of uptake trials over a period of time. To illustrate this, the linear uptake of Mn^{2+} from Mn^{2+} concentrations in the range 10 to 50 μM over a period of 7 min is shown in Figure 7.6.1. As all that is required from this data is the average uptake rate at each solute concentration, in all subsequent kinetic plots this experimental data will not be displayed, however it of the same nature as has been exemplified in Figure 7.6.1.

When data describing the initial uptake rates for Mn^{2+} concentrations in the range 10 to 100 μM are plotted against solute concentration, the familiar Michaelis-Menten curve is obtained (Fig. 7.6.2.). At 100 μM Mn^{2+}, the asymptote to the plateau portion of the curve cannot yet be estimated to give V_{max}, indicating that saturation of the transport sites is not fully achieved at this concentration. The Lineweaver-Burk form of the plot is shown in Figure 7.6.3. and gives a mean apparent transport constant, K_t, of 65.3 μM Mn^{2+} (S.E.E. range 61.1 -70.7 μM) and maximal transport rate, V_{max}, of 0.27 μmol (g dry wt)$^{-1}$ min^{-1} [range 0.21 - 0.35 μmol (g dry wt)$^{-1}$ min^{-1}]. At Mn^{2+} concentrations upto 1 mM however, a deviation from linearity was evident when uptake rates from these Mn^{2+} concentrations were combined with the previous data and a biphasic Lineweaver-Burk was

obtained, revealing an apparent low affinity site (Fig. 7.6.4.). When these data points are plotted separately by the Lineweaver-Burk method (Mn^{2+} concentrations in the range 0.2 - 1.0 mM) an apparent dissociation constant of 2.23 mM Mn^{2+} (range 2.09 - 2.40 mM) was obtained. This is shown in Figure 7.6.5.

It was observed in Chapter 6 that Mn^{2+} uptake from a relatively high concentration (50 μM) appeared to be unspecific, being inhibited by a number of other divalent cations present at a concentration of 100 μM. To investigate the nature of this inhibition, uptake rates from Mn^{2+} concentrations in the range 10 to 50 μM were determined in the presence and absence of 100 μM additions of Mg^{2+}, Co^{2+} and Zn^{2+} salts (Fig. 7.6.6.). Inhibition of Mn^{2+} uptake by these cations appeared to be competitive with the V_{max} remaining constant, giving apparent K_t values for Mg^{2+}, Co^{2+} and Zn^{2+} of 64 μM, 100 μM and 184 μM respectively as calculated from the slope of the line showing inhibition.

7.6.2. Kinetics of uptake from low manganese concentrations

The uptake kinetics were determined from the linear rates of uptake over 10 min from $^{54}Mn^{2+}$ concentrations in the range 2.5 to 50 nM. The Michaelis-Menten form of the plot indicated that transport site saturation had not occurred at 50 nM Mn^{2+} (Fig. 7.6.7.) and the Lineweaver-Burk plot of the data, shown in Figure 7.6.8., gave a mean value for K_t of 16.4 nM Mn^{2+} (range 12.3 - 24.4 nM) and for V_{max} of 1.01 nmol (g dry wt)$^{-1}$ min^{-1} [range 0.76 - 1.50 nmol (g dry wt)$^{-1}$ min^{-1}]. Since Zn^{2+}, at 10 μM, appeared to inhibit uptake by over 40 % and was one of the more inhibitory of the cations tested (see Section 6.3.2.), ^{54}Mn uptake was examined over the range 10 to 50 nM in the presence of 100 μM Zn^{2+}. The initial uptake rates were

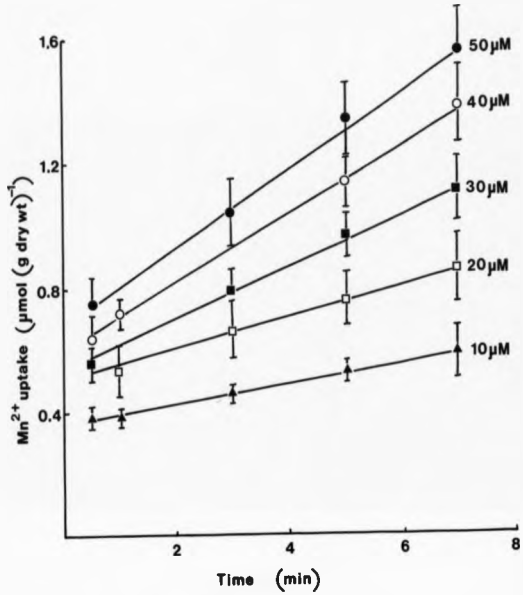


Figure 7.6.1. Linear uptake of Mn^{2+} from Mn^{2+} concentrations in the range 10 - 50 μM over the initial 7 min following metal addition.

Values represent the mean ± SE of 3 determinations.

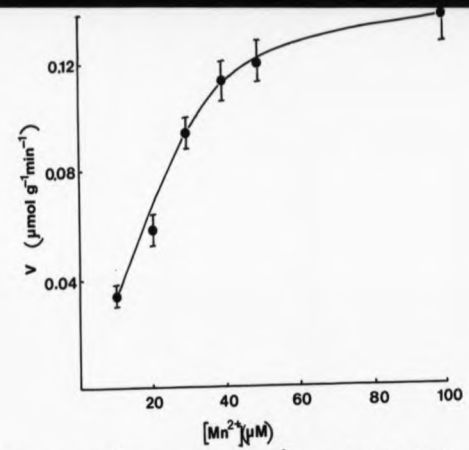


Figure 7.6.2. Initial Mn^{2+} uptake rates from Mn^{2+} concentrations in the range 10 - 100 μM. Values represent mean ± SE of 3 expts.

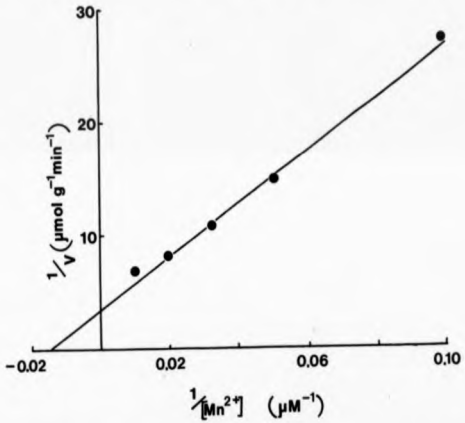


Figure 7.6.3. Lineweaver-Burk plot for Mn^{2+} uptake from concentrations in the range 10 - 100 μM Mn^{2+}

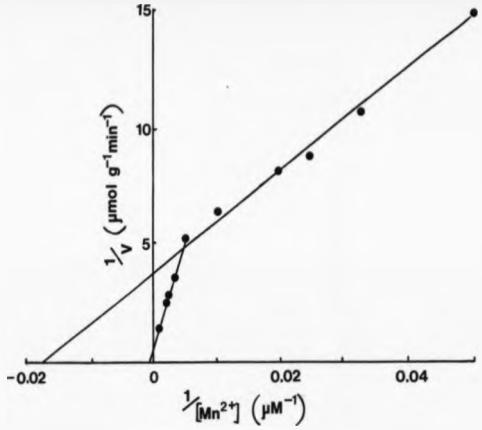


Figure 7.6.4. Lineweaver-Burk plot for Mn^{2+} uptake from concentrations in the range 20 μM - 1 mM, showing biphasic kinetics

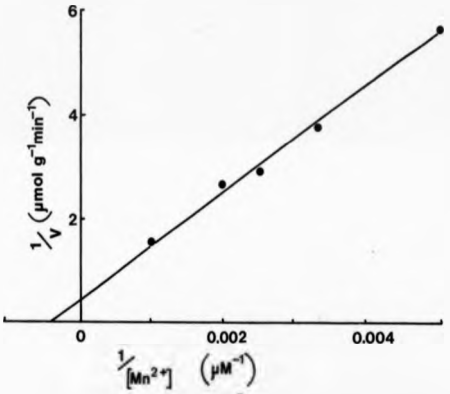


Figure 7.6.5. Lineweaver-Burk plot for Mn^{2+} uptake from concentrations in the range 0.2 - 1.0 mM Mn^{2+}

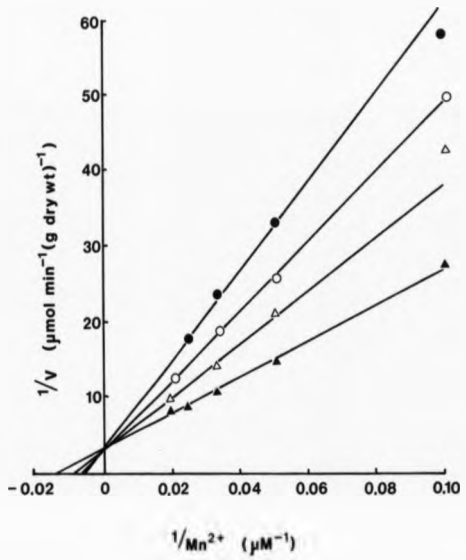


Figure 7.6.6. Lineweaver-Burk plot showing the kinetics of inhibition of Mn^{2+} uptake by 100 μM additions of Mg^{2+} (●); Zn^{2+} (△); Co^{2+} (○). A control (▲) was carried out with no additional divalent metals. Values represent means of at least 3 determinations.

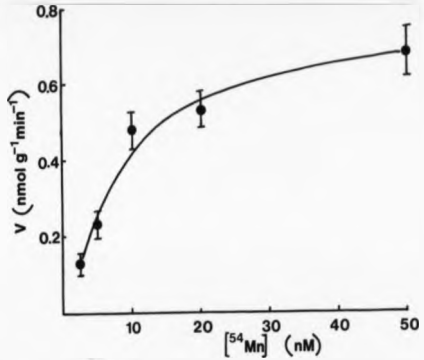


Figure 7.6.7. Initial ^{54}Mn uptake rates for ^{54}Mn concentrations in the range 2.5 - 50 nM. Values represent mean ± SE of 3 expts.

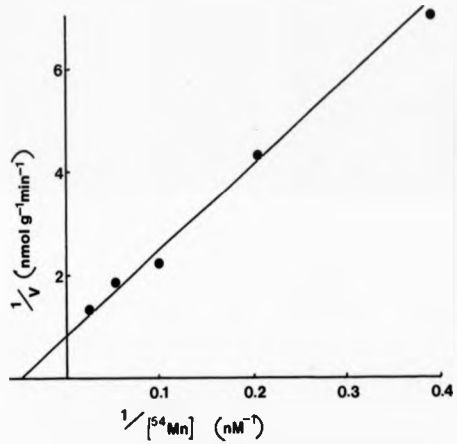


Figure 7.6.8. Lineweaver-Burk plot for ^{54}Mn uptake from concentrations in the range 2.5 - 50 nM ^{54}Mn.

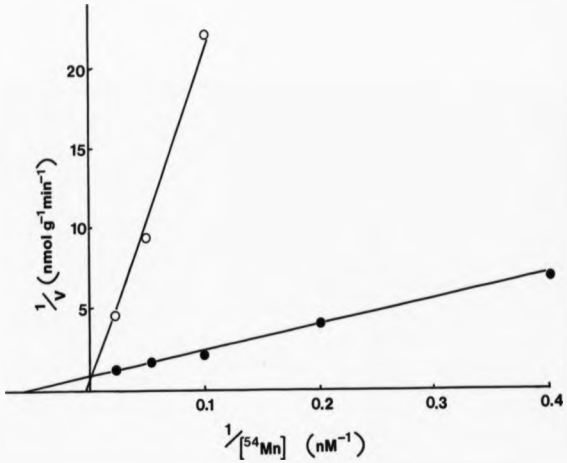


Figure 7.6.9. Lineweaver-Burk plot of Zn^{2+} inhibition of ^{54}Mn uptake. Uninhibited (●), inhibited by the addition of 100 μM Zn^{2+} (○). Values represent the means of at least 3 determinations.

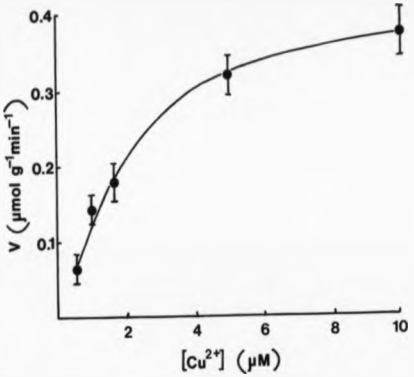


Figure 7.6.10. Initial uptake rates for Cu^{2+} concentrations in the range 0.5 - 10 μM. Values represent mean ± SE of 3 experiments.

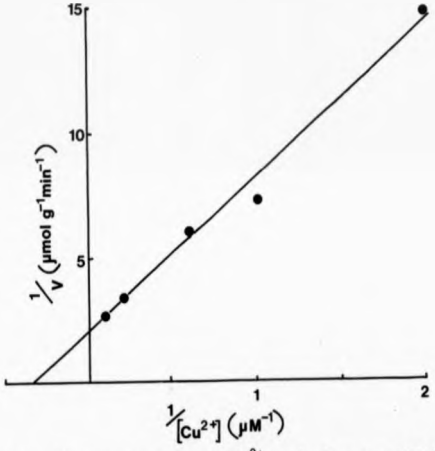


Figure 7.6.11. Lineweaver-Burk plot for Cu^{2+} uptake from concentrations in the range 0.5 - 10 μM Cu^{2+}.

plotted as before to determine the nature of this inhibition (Fig. 7.6.9.). Inhibition was competitive giving an inhibition constant, K_i, of 8.0 μM (range 7.3 - 8.9 μM).

7.6.3. Kinetics of copper uptake

Figure 7.6.10. describes the curve obtained when the Cu^{2+} uptake rates from Cu^{2+} concentrations in the range 0.5 to 10 μM were determined over a period of 5 min and plotted by the method of Michaelis-Menten. It is noted that after approximately 4 to 5 min the Cu^{2+} uptake rate decreased somewhat with subsequent uptake becoming non-linear. Hence, to ensure the measurement of linear uptake rates, all determinations were made during this initial period. The Lineweaver-Burk form of plot is shown in Figure 7.6.11. and gives a mean K_t value of 3.1 μM Cu^{2+} (range 2.5 - 3.9 uM) and V_{max} of 0.50 μmol (g dry wt)$^{-1}$ min^{-1} [range 0.37 - 0.66 μmol (g dry wt)$^{-1}$ min^{-1}].

7.7. Discussion

For the purposes of discussing the present results in the light of previous reports of metal transport kinetics, a table has been compiled which lists the K_t, V_{max} and competitive inhibitors of various microbial divalent cation transport systems (Table 7.7.1.). The V_{max} values from these studies are not complete as, in a number of reports, they have not been quoted as being on a dry weight basis and have been based on cell number or cell protein. In these instances, the value has been omitted as it provides no useful comparison.

Table 7.7.1. Kinetic parameters of various microbial metal transport systems

K_t (μM)	V_{max} (μmol min^{-1} g^{-1})	Competitive inhibitors (K_i, μM)	Species	Ref.
Mg^{2+}				
55	1.8	Co,270; Mn,250; Fe,390	*Rhod.capsulata*	[1]
50		Co,400; Mn; Ni	*E.coli*	[2]
18 (Tris-glucose)		Mn,500	"	[3]
31 (tryptone broth)		Mn,2000	"	[3]
30 (COR system)		Co; Mn; Ni	"	[4]
30 (MGT system)		Mg-specific	"	[4]
70	9.6		*Staph.aureus*	[5]
250	4.4	Mn,500	*Bac.subtilis*	[5]
300		Co; Mn; Ni; Fe	*Euglena gracilis*	[6]
20			*Sac.cerevisiae*	[7]
4000			"	[7]
Mn^{2+}				
0.5	0.02	Co,100; Fe,100	*R.capsulata*	[1]
0.2	0.02	Co,20; Fe,50	*E.coli*	[8]
1.1	0.1	Cd,3.4	*B.subtilis*	[9]
1.2	0.25-1.3	Mn-specific	"	[10]
2.0	0.06	Cd,1-8	*S.aureus*	[6]
0.2		Cd,0.9	*Lacto.plantarum*	[11]
< 10		Mg; Zn; Co; Ni	*S.cerevisiae*	[12]
100			"	[13]
1000			"	[14]
8.6×10^6			"	[15]
Co^{2+}				
200		Mg,10	*E.coli*	[2]
< 10	0.08	Mg; Mn; Zn; Ni	*S.cerevisiae*	[12]
77	5.4	Mg,125; Zn; Mn; Ni	"	[16]
100			"	[13]
100	0.16		"	[17]
800	1.05		"	[17]
10^5	200		"	[16]
1610		Mg	*Neurosp.crassa*	[18]
Cu^{2+}				
10		Zn	*Debaryomyces sp.*	[19]
70	3.2		"	[20]
220	0.22	Cd	*Aureo.pullulans*	[21]
390			*Pen.ochrochloron*	[22]

Table 7.7.1. (Continued)

Zn^{2+}				
20	2.7		*E.coli*	[23]
5.7-8.7	0.015		*Chlorella fusca*	[23]
0.36	2.2	Zn-specific	*Candida utilis*	[24]
1.3	0.21	Zn-specific	*Candida utilis*	[25]
20	0.1		*Asp.parasiticus*	[26]
90	0.51		*Spor.roseus*	[27]
< 10		Mg; Mn; Co; Ni	*S.cerevisiae*	[12]
1150	0.043		"	[28]
5000	9.09		"	[27]
200		Mn	*Neo.vasinfecta*	[29]
Ca^{2+}				
17			*B.subtilis*	[30]
1.9	0.004	Mg,3; K,800	*S.cerevisiae*	[31]
150			"	[32]
45		Mg,80; Co,92; Mn,106; Sr,130	*Schizosac.pombe*	[33]
300			"	[33]
Ni^{2+}				
100			*S.cerevisiae*	[13]
500		Mg; Mn; Zn; Co	"	[12]
Cd^{2+}				
1.8	1.53	Mn,1.2	*B.subtilis*	[9]
100	0.15	Ca,240	*A.pullulans*	[34]
1000	50	Mg,1400	*S.cerevisiae*	[16]

References

[1] Jasper & Silver, 1978
[2] Nelson & Kennedy, 1971
[3] Silver & Clark, 1971
[4] Park *et al*, 1976
[5] Jasper & Silver, 1977
[6] Silver, 1978
[7] Borst-Pauwels, 1981
[8] Silver *et al*, 1970
[9] Laddaga *et al*, 1985
[10] Eisenstadt *et al*, 1973
[11] Archibald & Duong, 1984
[12] Fuhrmann & Rothstein, 1968
[13] Norris & Kelly, 1979
[14] Rothstein, 1958
[15] Bianchi *et al*, 1981
[16] Norris & Kelly, 1977
[17] Heldwein *et al*, 1977
[18] Venkateswerlu & Sastry, 1970
[19] Wakatsuki *et al*, 1979
[20] Wakatsuki *et al*, 1985
[21] Gadd & Mowll, 1985
[22] Gadd & White, 1985
[23] Failla, 1977
[24] Lawford *et al*, 1980
[25] Failla *et al*, 1976
[26] Failla & Niehaus, 1986
[27] Mowll & Gadd, 1983
[28] Ponta & Broda, 1970
[29] Paton & Budd, 1972
[30] de Vrij *et al*, 1985
[31] Borbolla & Pena, 1980
[32] Pena, 1978
[33] Boutry *et al*, 1977
[34] Mowll & Gadd, 1984

The table shows the wide variation of kinetic parameters which have been obtained, with some apparent affinity constants varying over an order of magnitude for the uptake of a particular metal in the same species (see, for example, Zn^{2+} uptake in *S.cerevisiae*). Undoubtedly, differing experimental techniques, media composition and intraspecific variations will lead to a heterogeneity of affinity constants being obtained by different laboratories. With the effect on apparent kinetics of factors such as surface potential and complexation in mind, the result of using complex media for uptake studies can be clearly seen in the following example. Silver (1969) estimated a K_t value for Mg^{2+} transport in *E.coli* of 500 μM whilst Lusk and Kennedy (1969) obtained a value of 4 μM. This large discrepancy was found to be mainly due to the dependence of the K_t on the suspending media. Silver used tryptone broth to determine the K_t whilst Lusk and Kennedy employed a Tris-casamino acids medium. Subsequent determinations yielded values for K_t of 18 μM in a Tris-glucose medium and 31 μM in a more dilute tryptone broth (Table 7.7.1.). This illustrates the need to select the media constituents and buffer strength carefully for uptake experiments. The present series of experiments employed a simple non-complexing buffer which was used at a constant concentration throughout the study, hence kinetic deviations of the above type will be largely avoided yielding directly comparable data. However, despite the apparent diversity of results compiled in Table 7.7.1., a number of trends will emerge as each of the transport systems presently examined in *C.utilis* is discussed.

The apparent half-saturation constant of 65.3 μM Mn^{2+}, obtained from Mn^{2+} concentrations in the range 10 to 100 μM, is not dissimilar to a K_t of 100 μM for Mn^{2+} transport from the same concentration range in

S.cerevisiae (Norris & Kelly, 1979). Fuhrmann and Rothstein (1968), whilst unable to accurately determine the K_t for Mn^{2+} uptake in the same species, suggested it was probably below 10 μM. It is difficult to assess the relative capacity of the uptake system as indicated by the V_{max}, as no comparative studies in yeasts have been undertaken although the value seems rather high in comparison to bacterial studies of Mn^{2+} uptake. In addition, the usefulness of this value will be discussed shortly as it is generally found that, whilst the K_t may remain relatively constant within a given study, the V_{max} is subject to a great degree of variability depending on cell pretreatments, the environmental conditions and the physiological state of the cells (Silver, 1978).

There is little data available for yeast cells describing the kinetics of inhibition of this system by other divalent cations as most studies have simply reported the gross inhibition of Mn^{2+} uptake by divalent salts over a period of time. It should be noted that a metal can reduce uptake of another in a number of ways other than by direct competitive effects (Norris & Kelly, 1979), however the present results show strong competitive inhibition of Mn^{2+} uptake with affinities for the transport sites being Mn^{2+} and $Mg^{2+} > Co^{2+} > Zn^{2+}$. It appears therefore that these cations share the same transport system at the concentrations studied. The relative affinities of the cations for the transport sites in this kinetic study agree with the affinity series obtained in Chapter 6 by measuring the total uptake from 50 μM Mn^{2+} after 20 min. The only data of a similar nature reported in *C.utilis* (Fencl *et al*, 1974) was not obtained from kinetic measurements but from the total uptake following 2 h incubation in non-equivalent metal concentrations and gave a high affinity for Zn^{2+}.

Bianchi *et al* (1981) have stated that the presence in *S.cerevisiae* of a low affinity, high capacity Mn^{2+} transport system is difficult to reconcile with the fact that Mn^{2+} is required in such small amounts by the yeast cell. Indeed it is more likely that this transport system is involved in the control of the intracellular level of Mg^{2+} and this is supported by the observed high affinity of Mg^{2+} for this system. Thus it is not unreasonable to assume that the system of broad specificity studied is the primary Mg^{2+} transporter and that Mn^{2+} can fortuitously enter the cell as a substrate of equal or slightly lower affinity than Mg^{2+} and with Co^{2+} and Zn^{2+} exhibiting less affinity. Hence the kinetic parameters obtained in this study compare favourably with the reported values of K_t and K_i for the Mg^{2+} transporters of a number of microbial cells (Table 7.7.1.). Bacteria, which appear to have the most comprehensively studied Mg^{2+} uptake systems, have transport constants well within an order of magnitude of the value reported here. With these species, Mg^{2+} uptake was competitively inhibited by Co^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+} and probably Zn^{2+} (Silver, 1978). The table shows that the competitive inhibitors had lower affinities than Mg^{2+}, the K_i values being generally 4 to 10 times higher than the K_t for Mg^{2+}. The system observed here in *C.utilis* did not appear to exhibit quite the same degree of specificity: the affinity of Mg^{2+} for the transport site was approximately the same as Mn^{2+} and was 1.5 and 2.8 times greater than for Co^{2+} and Zn^{2+} respectively. The universality of this low specificity Mg^{2+} uptake system is reflected in the compiled data for the transport kinetics of various other divalent cations presumably entering *via* this system, particularly the most widely studied cations; Co^{2+}, Zn^{2+}, Ca^{2+} and Ni^{2+} (Table 7.7.1.). In the majority of cases where the K_t is greater than around 10 μM, the system exhibits comparatively little specificity for the cation studied.

The biphasic Lineweaver-Burk plot obtained at Mn^{2+} concentrations up to 1 mM and giving an affinity constant of 2.23 mM has been observed in several previous studies of *S.cerevisiae*. Affinity constants for Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and Cd^{2+} of 4, 1, 100, 1.15 and 1 mM respectively have been reported (Table 7.1.1.) and Borst-Pauwels (1981) has emphasised the presence of low and high affinity sites in yeast cells. Norris and Kelly (1977), examining Co^{2+} uptake in *S.cerevisiae*, detected a sharp reduction in affinity and an increase in the rate of Co^{2+} uptake at concentrations above 5 mM Co^{2+} whilst Wakatsuki *et al* (1979) observed a similar effect for Cu^{2+} uptake in *Debaryomyces hansenii* at Cu^{2+} concentrations in the range 0.1 to 5.0 mM although no data was given as to the viability of *D.hansenii* at these high Cu^{2+} concentrations. These results apparently show that cations bind to the defined sites having high affinity at low concentrations, and at high concentrations, metal ions bind to not only the high affinity sites but also to various low affinity sites.

It is not possible from this data to determine whether the different affinities for divalent cation uptake reflect a change in the translocation process of a single uptake system or the presence of two different transporters. Borst-Pauwels (1981) has suggested several alternative possiblities. A two-site translocation process may be involved similar to the monovalent cation uptake system consisting of a transport site and a modifier site. Complex formation with buffer anions may lead to an increase in the apparent dissociation constant as may adsorption of the cation to the cell wall or membrane. This latter suggestion seems more likely in the light of a report that the greater of two affinity constants for Ca^{2+} uptake in *Sch/izosaccharomyces pombe* pertained to glucose-independent Ca^{2+} accumulation (Boutry *et al*, 1977). At high solute

concentrations, it is expected that the membrane would be depolarised due to high intracellular accumulation of cations (Borst-Pauwels, 1981). Thus, deviations from Michaelis-Menten kinetics, manifested by an increase in the apparent cation-transport site dissociation constant and experienced under conditions of high cation concentrations may be due to the reduced negative membrane potential.

One further explanation for transport kinetic deviations may be the involvement of a changing surface potential with increasing solute concentration. As previously explained, a decrease in surface potential concomitant with an increase in the bulk monovalent cation concentration gives rise to an increase in the K_t value as the apparent affinity of the divalent cation for the transport site decreases. However the converse is also true, as the divalent cation concentration is raised, the divalent cations will displace monovalent salts from the diffuse double layer, thus increasing the effective concentration of divalent cations at the membrane surface and increasing the translocation rate. Hence a decrease in the K_t results (Theuvenet & Borst-Pauwels, 1977). Whilst it is as yet difficult to estimate the relative importance of the effects of the surface potential as the dependence of surface potential on divalent cation concentration is not accurately known, it is clear that the above explanation is incompatible with the observed results. The apparent transport constant actually increased from 65.3 μM to over 2 mM and so it can be concluded that the contribution of the surface potential to the kinetic deviations observed is probably of minor significance in the present experimental system.

It was noted earlier in this discussion that uptake from Mn^{2+} concentrations around 10 μM was relatively non-specific and was

probably *via* the Mg^{2+} transport system. Uptake from nanomolar Mn^{2+} concentrations proved to be highly specific and yielded a transport constant almost 4000-fold smaller than the K_t obtained for micromolar Mn^{2+} concentrations. It is believed that uptake from these trace concentrations is by way of a Mn^{2+}-specific micronutrient transport system. As far as the author is aware, the kinetic data available in the literature for Mn^{2+} uptake in yeasts pertains to uptake *via* the Mg^{2+} transporter and, as would be expected for such a system, gives affinity constants several orders of magnitudes greater than the K_t value of 16.4 nM Mn^{2+} observed in this study. The kinetic data from this system indicate that Mn^{2+}-specific uptake would become saturated and thus undetected at higher Mn^{2+} concentrations. The only comparable affinity constants calculated for micronutrient transport systems in yeasts are 0.36 μM Zn^{2+} (Lawford *et al*, 1980) and 1.3 μM Zn^{2+} (Failla *et al*, 1976) for Zn^{2+}-specific uptake in *C.utilis*. In the latter study, deviations from simple Michaelis-Menten kinetics came to the fore and several technical and methodological explanations were given. It is possible that, at the higher Zn^{2+} concentrations studied (around 10 μM), some uptake of Zn^{2+} *via* the Mg^{2+} transport system may have occurred once the Zn^{2+}-specific transporter became saturated. However, in bacteria, K_t values for Mn^{2+}-specific transport ranging from 0.05 to 2 μM Mn^{2+} have been reported (Silver & Kralovic, 1969, Silver, 1978, Archibald & Duong, 1984). The maximal Mn^{2+} uptake rate in this strain of *C.utilis* [1.01 nmol (g dry wt)$^{-1}$ min^{-1}] is somewhat lower than in previously described systems (Silver, 1978), and this may reflect a difference in Mn^{2+} requirements between various cell types or variations in cell pretreatments.

Zn^{2+} competitively inhibited Mn^{2+} uptake; however the inhibitory constant, K_i, was some 500 times greater than the K_t for Mn^{2+} indicating a similar level of specificity as Mn^{2+}-specific transport in bacteria (Table 7.7.1.). It is probable that the other cations might also exert some reduced competitive effect as it is unlikely that the specificity will be complete in a mechanism of this type. Cd^{2+} has been shown to inhibit competitively Mn^{2+} uptake in *Bacillus subtilis*, *Staphylococcus aureus* and *Lactobacillus plantarum* (Table 7.7.1.), having an inhibitory constant similar to, or greater than, the Mn^{2+} transport constant. This may well be a reflection of the fact that this highly toxic cation is not generally present, except as a pollutant, in the normal physiological environment and by virtue of its similar physico-chemical characteristics to Mn^{2+} may fortuitously enter the cell through the specific Mn^{2+} uptake system. For reasons outlined in the preceding chapter, Cd^{2+} was not included in this study.

In a treatise on the kinetic considerations of membrane transport, Christensen (1975) has stated that investigations of solute uptake are often interrupted at unecessarily low or high concentrations and that once one mediated system has been observed, any other system with a lower or higher K_t is often overlooked. With the exception of Zn^{2+} uptake in *C.utilis*, this appears to have been the case for divalent cation translocation in yeasts. Microorganisms often have heterogeneous transporter types for a given solute and, unless their Michaelis constants differ by an order of magnitude or so, these systems can be difficult to distinguish kinetically (Button, 1983). Fortunately in the present study, the constants for Mn^{2+} transport *via* the Mg^{2+} uptake system and the Mn^{2+}-specific carrier differed by over 3 orders of magnitude. However, one problem of evaluating

kinetic data from such a multicomponent process is that the sum effect of both transport systems will be measured at intermediate concentrations between the solute concentration ranges for each system. Hence if two systems with similar affinities are analysed, this overlap effect will give a net measurement of the two systems, introducing considerable error into the kinetic constants (Christensen, 1975). The error is likely to be minimal in the current study due to the great dissimilarity of the transport constants for the two Mn^{2+} uptake systems. At transport site saturation for Mn^{2+}-specific uptake, transport *via* this system would account for only 0.37 % of the total Mn^{2+} uptake rate at the maximal capacity of the Mg^{2+} transport system. At 10 % of the V_{max} of Mn^{2+} uptake *via* the Mg^{2+} carrier (which corresponds to the lower limit of the concentration range studied: 10 μM), Mn^{2+}-specific uptake only accounts for 4 % of the observed uptake rate, which is within the standard error of estimate of the rate constant obtained.

The transport constant for Cu^{2+}-specific uptake of 3.1 μM is similar to a K_t of 1.3 μM for Zn^{2+}-specific transport in the same species (Failla *et al*, 1976). Whilst the V_{max} is twice as great as the capacities of both the Zn^{2+} transporter and the Mg^{2+} system for Mn^{2+} observed in this study, it was generally noted that the initial Cu^{2+} uptake rate in *C.utilis* was markedly greater than those for other divalent cations. It is interesting to note that one previous study of Cu^{2+} uptake in *C.utilis* (Khovrychev, 1973)reported an absorption constant of 1.2 mM and maximum absorption rate around 0.8 to 1.0 μmol g^{-1} min^{-1}. However the Cu^{2+} concentration range studied was from 0.5 to 2.7 mM Cu^{2+} and no mention was made of Cu^{2+} toxicity. The results from Chapter 4 demonstrate that Cu^{2+} toxicity is considerable in *C.utilis* at concentrations as low as 20 μM, suggesting that the

previous author may have been observing adsorption to cell surfaces and penetration into the interior of dead cells as opposed to energy-dependent transport. In addition, no energy source appears to have been added to the uptake medium and Cu^{2+} uptake was measured, not by cellular accumulation, but by disappearance of Cu^{2+} from the buffer solution thus preventing differentiation of the intracelluar Cu^{2+} component from the non-specifically surface-bound component.

The only other kinetic constants for Cu^{2+} uptake in yeasts and fungi are reported in *D.hansenii*, *Aureobasidium pullulans* and *Penicillium ochro-chloron* (Table 7.7.1.). *A.pullulans* and *P.ochro-chloron* are both Cu^{2+} tolerant fungi and, as such, may be expected to possess a much lower affinity for Cu^{2+} transport. The results of the study of Wakatsuki *et al* (1979) on Cu^{2+} absorption in *D.hansenii* also have very limited comparative value; as no energy source was present in the incubation medium and millimolar concentrations of Cu^{2+} were employed, the study appears to be largely one of energy-independent Cu^{2+} binding. Furthermore, in an earlier report by Imahara *et al* (1978), this same strain of *D.hansenii* was selected for study due to its sensitivity to Cu^{2+}.

A summary of the kinetic constants obtained for *C.utilis* in this study is given in Table 7.7.2. The affinity constant for Mn^{2+} binding to the very low affinity site (2.23 mM) has been excluded from this table as the site has not been fully characterised. Its presence was only detected in gratuitous Mn^{2+} concentrations far in excess of those normally experienced under normal physiological conditions and it is probably of minor importance to the cell. As mentioned earlier, the kinetic parameters of various cation transport systems are often related to the cellular requirement for each cation

Table 7.7.2. Summary of transport kinetic data obtained in *C.utilis*

Metal & transport system studied	Uptake conc. range	K_t (μM)	V_{max} (μmolg^{-1}min^{-1})	Competitive inhibitors, K_i (μM)
Mn^{2+} (via Mg^{2+} system)	10 - 100 μM	65.3	0.27	Mg^{2+}, 64 Co^{2+}, 100 Zn^{2+}, 184
Mn^{2+} (via Mn^{2+}-specific system)	2.5 - 50 nM	0.0164	0.001	Zn^{2+}, 8.0
Cu^{2+} (via Cu^{2+}-specific system)	0.5 - 10 μM	3.1	0.50	

(Jasper & Silver, 1977). It can be seen from Table 7.7.2. that the transport system for Mg^{2+}, an abundant cation for which the cell has a high requirement, has a low affinity and relatively high V_{max}, whereas the Mn^{2+}-specific system is a micronutrient transport system operational at extremely low Mn^{2+} levels and with a low V_{max}. As Mn^{2+} is present in the external milieu at such trace concentrations, the purpose of this transport system would be to scavenge this metal for which the cell has an essential requirement. This can also be seen, albeit less dramatically, for the Cu^{2+} transport system which in terms of its K_t, bears a strong resemblence to Zn^{2+} transport also reported in *C.utilis*. Thus a transport system of very high affinity may be required for Mn^{2+} uptake. Indeed, microorganisms which possess a low K_t for solute transport are thought to have a special advantage in low-substrate environments (Button, 1985).

Whilst the kinetic data in this study has facilitated characterisation of each transport system examined here, the interpretation of kinetic constants is not always clear. Rates of solute transport by microorganisms are difficult to determine. Systematic problems including organism growth, damage or de-energisation of transport systems during cell collection, transport induction and binding, partitioning or depletion of the substrate can all affect transport constants and their effect should be recognised (Button, 1983). Changes of the growth-phase status of cells and the medium pH have been shown to directly affect the V_{max} of transport systems as has whether the cells are energy-rich or energy-deficient (Nelson & Kennedy, 1971, Silver, 1978, Gadd & White, 1985). Hence it is emphasised that the kinetic constants obtained are apparent kinetics based on empirical observations under the conditions imposed and should not be taken as absolute values.

Because of these limitations, any meaningful comparisons of transport parameters should be confined to studies of K_t (Lerner & Larimore, 1986). If uptake conditions are not identical, any subsequent conclusions that the systems are separate should only be drawn if the values differ by at least an order of magnitude.

The introductory section of this chapter emphasised the need for a comparative examination of the various existing transport systems under standard conditions. It has been shown in *C.utilis* that at least four distinct uptake mechanisms exist to provide the cell with essential divalent metals; the low specificity Mg^{2+} transporter (which is also capable of transporting other divalent cations), the specific uptake systems for Mn^{2+} and Cu^{2+} described here and Zn^{2+}-specific system reported by Failla *et al* (1976). Their kinetic values have now been defined and can be compared directly, as can the inhibitory effect of other metals on these systems. The physiological importance of these processes has been stressed in Chapter 6. Now that these systems have been outlined, the remaining experimental chapters are aimed at further characterising the cell-metal interactions involved with each of these systems and examining the cellular regulation of such systems.

8. DIVALENT CATION EXIT-EXCHANGE REACTIONS

8.1. Introduction

In any study of uptake of solute into a cell, it is important to be aware of subsequent efflux of the particular solute from the cell back into the external milieu. If this efflux is large and is concomitant with influx, the apparent uptake kinetics would clearly differ from the actual kinetic data. In addition, the movement of a cation into a cell will lead to the movement of an equivalent number of cations in the opposite direction in order to maintain the ionic balance (Jennings, 1963). In this chapter, these two facets of ion transport will be examined in relation to bivalent cation uptake in *C.utilis*.

As well as possessing systems for the uptake of metals, many microorganisms have transport systems to excrete certain cations from the cell. This appears to be an important way in which cells may regulate the intracellular concentrations of metals. In addition to the multitude of cations which must be concentrated within the cells for growth, there are also unessential yet abundant cations whose intracellular concentrations must be kept low in comparison to the concentrations in common growth media (Silver, 1978). Examples of these cations are Ca^{2+} and Na^{+} and, for this purpose, microbial cells have evolved specific, energy-dependent egress systems which have properties in many respects similar to metal uptake systems. Ca^{2+} egress systems have been observed in *Escherichia coli*, *Bacillus megaterium*, *Rhodopseudomonas capsulata* and *Streptococcus faecalis* (Silver, 1978) and in *Bacillus subtilis*, energy-dependent uptake of Ca^{2+} was observed in inside-out membrane vesicles whilst Ca^{2+} efflux

was seen in right-side-out orientated vesicles (de Vrij *et al*, 1985). A Ca^{2+}/H^{+} antiport system was thought to responsible in the latter species. Indeed, there is now sufficient evidence to suggest that a Ca^{2+} extrusion system is a general characteristic feature of all bacterial cells. There is much less data available for yeasts. Boutry *et al* (1977) described Ca^{2+} efflux in *Schizosaccharomyces pombe* which was linear with respect to time, unaffected by Ca^{2+} in the external medium and dependent on the glycolysis of endogenous substrate for its operation. Ca^{2+} efflux also occurred in *Saccharomyces cerevisiae* where it appeared to be tightly coupled to K^{+} influx, however efflux could also be induced by other mono- or divalent cations (Eilam, 1982).

Outwardly orientated transport systems have also evolved to prevent cellular build-up of inhibitory levels of toxic ions. Cu^{2+} efflux from Cu^{2+}-loaded cells of the dinoflagellate, *Gonyaulax tamarensis*, occurred over a long time period to achieve a constant cellular Cu^{2+} quota (Schenk & Hull, 1985). The authors suggested that this observation was compatible with the induction of a Cu^{2+} efflux pump although this possibility was not tested. The role of a highly efficient chemiosmotic efflux system specific for Cd^{2+} has been implicated in Cd^{2+} resistance in *Staphylococcus aureus* (Trevors *et al*, 1985). Resistant cells preloaded with Cd^{2+} completely effluxed Cd^{2+} when transferred to Cd^{2+}-free buffer.

Efflux of essential accumulated metal cations has also been observed widely in bacteria and to a much lesser extent in yeasts. Efflux of Mg^{2+} from cells of *E.coli* in the presence of external Mg^{2+} was temperature and energy-dependent and was indicative of a carrier-mediated process (Silver & Clark, 1971). The rates of uptake

and loss of Mg^{2+} varied with the external Mg^{2+} concentration such that the cell Mg^{2+} content remained relatively constant. The cations, Co^{2+}, Mn^{2+} and Ni^{2+} and non-radioactive Mg^{2+}, which could be transported by the Mg^{2+} uptake system promoted release of accumulated $^{28}Mg^{2+}$ (Nelson & Kennedy, 1971). Ca^{2+}, which has a very low affinity for the Mg^{2+} influx system of *E.coli*, had no effect on Mg^{2+} efflux. There appeared to be a direct relationship between uptake and efflux in these studies: increasing the Mg^{2+} uptake rate two-fold increased Mg^{2+} release also by a factor of two, and the half-saturation concentration for added external Mn^{2+} promoting Mg^{2+} efflux was similar to the K_i for Mn^{2+} as a competitor of the Mg^{2+} uptake system. Furthermore, Co^{2+} had no effect on Mg^{2+} efflux in mutant cells which were defective in the Co^{2+}-Mg^{2+} transport system.

In a useful review of microbial Mg^{2+} transport, Jasper and Silver (1977) have outlined the current picture of Mg^{2+} transport and efflux in *E.coli* and this is illustrated in Figure 8.1. The diagram shows how, at step 1, Mg^{2+} or other divalent cations of a lower affinity enter the cell *via* the Mg^{2+} uptake system. Inside the cell, it is estimated that upto 90 % of the total cell Mg^{2+} may be associated with ribosomal material. Newly accumulated Mg^{2+}, Mn^{2+} or Co^{2+} (and possibly Ni^{2+}) displaces cell Mg^{2+} from its ribosomal sites (step 2) which increases Mg^{2+} efflux through the Mg^{2+} transport system (step 4). In external Mg^{2+}-free conditions, Mg^{2+} released from the cell into the periplasmic space could be recaptured by the uptake system, however in high external concentrations of Mg^{2+}, Mn^{2+} or Co^{2+}, the Mg^{2+} released would be immediately diluted by these cations and be less likely to be reinternalised. Finally, intracellular Mn^{2+} or Co^{2+} may displace Mg^{2+} involved in membrane stabilisation (step 3) leading to non-specific localised cell leakage.

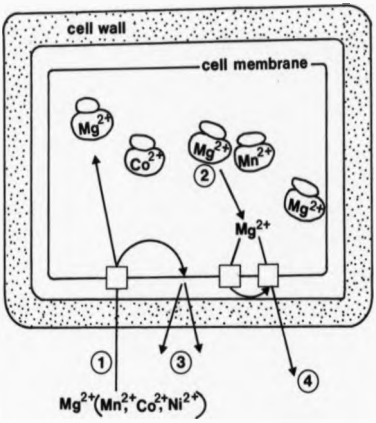


Figure 8.1. Model for transport and inhibitory effects of divalent cations. Steps: 1) uptake of various cations by the Mg^{2+} transport system; 2) displacement of Mg^{2+} from intracellular polyanions, primarily on ribosomes; 3) non-specific membrane leakage induced by Mn^{2+} or Co^{2+} from within; 4) egress of Mg^{2+} (or Mn^{2+}) by way of the Mg^{2+} transport carrier with "recapture loop" within the periplasmic space.
(Taken from Jasper & Silver, 1977)

Likewise, a similar mechanism operates for Mn^{2+} uptake and efflux in *E.coli* where about 80 % of the $^{54}Mn^{2+}$ accumulated is rapidly exchanged with non-radioactive Mn^{2+} upon resuspension in 10 μM Mn^{2+} (Silver & Kralovic, 1969, Silver *et al*, 1970). The Mn^{2+} is either free inside the cells or is bound to proteins or nucleic acids where it is readily dissociable (both Mg^{2+} and Mn^{2+} can competitively bind to ribosomes). Efflux from ^{54}Mn-preloaded cells has also been reported in the bacteria, *B.subtilis* (Eisenstadt *et al*, 1973) and *Lactobacillus plantarum* (Archibald & Duong, 1984). In a report on *B.subtilis*, Fisher *et al* (1973) showed that a 25-fold variation in the cellular Mn^{2+} content was due to a difference in the Mn^{2+} influx rate as the efflux rate remained constant throughout. The usefulness of such a system can be seen in cells which have an artificially high Mn^{2+} content. In such cells, protein and ribonucleic acid synthesis is prevented, however the Mn^{2+} efflux system subsequently releases upto 90 % of the total cell Mn^{2+} to relieve the inhibition and elicit growth (Fisher *et al*, 1973).

Information on efflux of metals other than Ca^{2+} from yeast and fungal cells is scant. The comparatively greater accumulation of Mn^{2+} than of Sr^{2+} in *S.cerevisiae via* a transport system of broad specificity has been ascribed to a faster rate for Sr^{2+} egress than for Mn^{2+} (Nieuwenhuis *et al*, 1981, Theuvenet *et al*, 1986). Release of Cu^{2+} in preloaded cells of *Aureobasidium pullulans* involved two phases of efflux; the first being rapid accounting for 36 % of cell Cu^{2+}, and the second being much slower with a half-life of 484 min and accounting for 64 % of the cell Cu^{2+} (Gadd & Mowll, 1985). This efflux was not dependent on the presence of either glucose or extracellular metal ions, unlike the other reports. Finally, energy-dependent Cd^{2+} efflux has been described in Cd^{2+}-loaded cells

of *A.pullulans* (Mowll & Gadd, 1984). It was not clear in these studies whether the metal under investigation was a low affinity substrate of the Ca^{2+} efflux system or whether some other egress system operated. However, in view of the similarities between Cd^{2+} and Ca^{2+} ions in terms of their physico-chemical characteristics, it seems quite likely that the latter study observed Cd^{2+} release *via* the putative Ca^{2+} efflux mechanism.

To maintain electroneutrality during cation uptake, a cell must concomitantly take up an anion or release a second cation. Exchanges of this type have been documented for metal uptake in a number of yeast and fungal species. Eddy and Hopkins (1985) have stated that in yeasts the uptake of positive charge can be neutralised in several ways: a spontaneous K^+ efflux may occur or protons may be ejected through the plasma-membrane ATPase.

Several reports have detailed K^+ release in divalent cation uptake. Unfortunately, one consequence of metal toxicity is the dramatic loss of cell K^+. K^+ released in this way should be carefully distinguished from K^+ efflux resulting from metal influx. For example, uptake of Cu^{2+}, Cd^{2+} and Zn^{2+} from toxic concentrations of these metals in *S.cerevisiae* led to extensive K^+ loss, presumably due to increased membrane permeability (Norris & Kelly, 1977, Mowll & Gadd, 1983, Gadd *et al*, 1984a, 1984b). In contrast to uncontrolled K^+ loss of this nature, stoichiometric exchanges in the ratio, 2 K^+ ions released for each divalent cation taken in, have been observed in yeasts. The maximal rate of K^+ efflux was exactly twice that of Cu^{2+} influx (Gadd & Mowll, 1985) or Cd^{2+} influx (Mowll & Gadd, 1984) in *A.pullulans*. This efflux was not symptomatic of membrane disruption and indicated a $2K^+ : Cu^{2+}$ or Cd^{2+} stoichiometry. The

former report showed that influx-efflux was tightly coupled as the ionophore nigericin, in inducing increased K^+ release, also increased Cu^{2+} uptake. Lichko *et al* (1980) observed Mn^{2+} uptake accompanied by K^+ loss in a 1 : 2 ratio in *Saccharomyces carlsbergensis* whilst other authors have reported the stoichiometric release of K^+ in exchange for the metals, Mn^{2+}, Ca^{2+} and Co^{2+} in *S.cerevisiae* (Fuhrmann & Rothstein, 1968, Norris & Kelly, 1977, Okorokov *et al*, 1979, Eilam *et al*, 1985b).

K^+ is not the only cation capable of compensating divalent cation uptake by its release. Na^+-loaded cells prepared by growth in Na^+-rich and K^+-deficient conditions, released 2 Na^+ ions for each Co^{2+} cation taken up (Fuhrmann & Rothstein, 1968). According to the chemiosmotic theory, microorganisms can accumulate solutes in response to proton extrusion and this appears to have been the case in the following examples. Roomans *et al* (1979) could detect no K^+ efflux on addition of Sr^{2+} or Ca^{2+} to a suspension of metabolising *S.cerevisiae* cells, but instead observed that, as in monovalent cation uptake, divalent cation uptake was coupled to H^+ efflux. Mg^{2+}/H^+ exchange has been observed in *S.cerevisiae* and in spinach chloroplasts (Conway & Beary, 1958, Jasper & Silver, 1977), and in plasma-membrane vesicles of *S.cerevisiae*, Mn^{2+} uptake was also balanced by an equivalent H^+ loss (Lichko *et al*, 1980). Furthermore, Ca^{2+}/H^+ antiport systems have been described in *Neurospora crassa* (Stroobant & Scarborough, 1979) and in the vacuolar membrane of *S.cerevisiae* (Eilam *et al*, 1985a) whilst Ca^{2+} uptake in *S.pombe* was driven by electrogenic proton extrusion (Boutry *et al*, 1977).

Hence the purpose of the experiments described in this chapter is two-fold; to investigate metal efflux and to examine any cation

exchanges to maintain electroneutrality during divalent cation uptake *via* the transport systems outlined in the preceding chapters.

8.2. Materials and methods

8.2.1. Divalent cation efflux

Cells were collected and prepared as described in Chapter 3 prior to experimentation. To preload cells with a divalent cation, cells were incubated in a buffer solution containing 50 mM MES and 50 mM glucose in the presence of ^{54}Mn, non-radioactive Mn^{2+} and Cu^{2+} at concentrations of 10 nM, 100 μM and 2.5 μM respectively. Cells suspended in ^{54}Mn and non-radioactive Mn^{2+} were incubated for 30 min whilst the cells in 2.5 μM Cu^{2+} were incubated for 15 min. Cells were then rapidly separated by membrane filtration, washed once with 2 mM $CaCl_2$ then three times with distilled water, and resuspended in MES buffer in the presence or absence of glucose as stated. The addition of metal salts was made at 22 min.

8.2.2. Monovalent cation - divalent cation exchange

Divalent cation uptake experiments were carried out in the manner previously described in Chapter 3. K^+ determinations of both cell samples and filtered buffer samples were made using atomic absorption spectroscopy. To analyse H^+ release, cells were resuspended into unbuffered 50 mM glucose solution and the pH maintained constantly at 5.50 ± 0.02 using a pH-stat (Pye-Unicam) by the addition of 10 mM NaOH as a titrant. Cell samples, placed in a 600 ml glass vessel, were maintained at 30 ^{o}C by a heated water-jacket, agitation being provided by a magnetically-coupled stirrer bar. The rate of H^+ release was determined from the equivalent amount of NaOH required to

maintain the pH at 5.5. The H^+ release rates were determined from the linear release over a period of 5 - 10 min.

8.3. Results

8.3.1. Divalent cation efflux

Figure 8.3.1. describes the effects of glucose and $MgCl_2$ on the cellular Mn^{2+}. As the initial cell Mn^{2+} concentration varied considerably between each batch of Mn^{2+}-preloaded cells, presumably due to experimental differences during uptake and cell washing procedures, the standard errors are of little use and have not been included on this figure. However, the results shown are the mean of three sets of data and the graph is typical for all batches of cells examined. Thus presenting the mean data in this way provides a clear reflection of the pattern of results obtained. In the absence of glucose the cellular Mn^{2+} level remained constant with no observed leakage of Mn^{2+} back into the buffer. A similar result was obtained when glucose had been added to the medium. However, when 5 mM $MgCl_2$ was added after 22 min, a fairly linear release of Mn^{2+} was observed at a rate of 20 nmol (g dry wt)$^{-1}$ min^{-1}.

The effect of the inhibitor CCCP, and external $MnCl_2$ on much lower cellular concentrations of Mn^{2+} than used in the previous experiment is shown in Figure 8.3.2. Cells preloaded with ^{54}Mn and resuspended into Mn^{2+}-free buffer containing glucose maintained a constant cell ^{54}Mn concentration. Likewise, the addition of CCCP had no effect on cellular ^{54}Mn. When 1 mM $MnCl_2$ was added at 22 min, 25 % of the cellular ^{54}Mn was released into the medium during the next 13 min followed by a somewhat slower release over the next 80 min. The ^{54}Mn

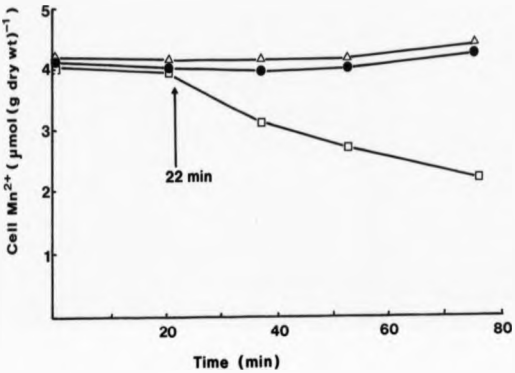


Figure 8.3.1. Cellular Mn^{2+} content of high-Mn^{2+}-loaded cells in the presence of glucose (●); in the absence of glucose (△); in the presence (□) of glucose with 5 mM $MgCl_2$ added at 22 min. Values represent the mean data of 3 separate determinations.

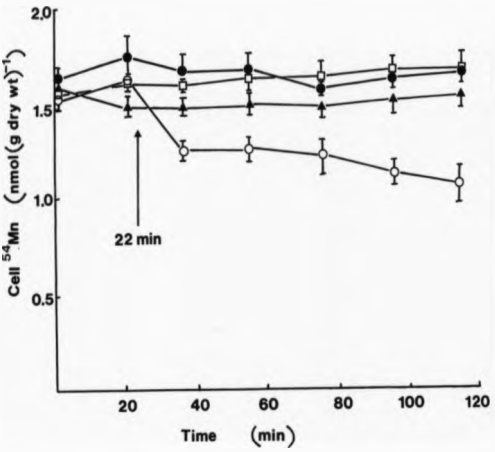


Figure 8.3.2. Cellular ^{54}Mn content of low-^{54}Mn-loaded cells in the absence (□,○) and presence (●,▲) of 100 μM CCCP, with the addition of 1 mM $MnCl_2$ at 22 min (▲,○). Values shown are the mean ± SE of 3 experiments.

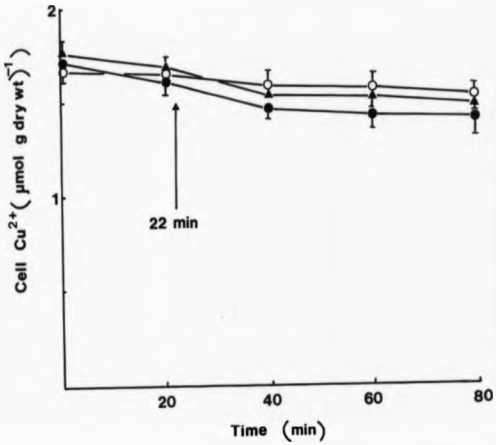


Figure 8.3.3. Cellular Cu^{2+} content of Cu^{2+}-loaded cells in the presence of glucose (●); in the absence of glucose (○); in the presence of glucose with 5 mM $MgCl_2$ added at 22 min (▲). Values represent the mean ± SE of 3 determinations.

egress rate was too slow to be accurately determined, however a total loss of 0.4 nmol ^{54}Mn (g dry wt)$^{-1}$ was recorded over 90 min. This release of ^{54}Mn was prevented by the addition of CCCP.

When cells preloaded with Cu^{2+} were resuspended in buffer either in the absence or presence of glucose, no Cu^{2+} efflux was observed. Similarly, on addition of 5 mM $MgCl_2$ at 22 min, there was no loss of cell Cu^{2+} (Fig. 8.3.3.).

8.3.2. Monovalent cation - divalent cation exchange

As *C.utilis* cells grown in normal media contain high intracellular K^+, cell digests had to be diluted by a factor of 100 - 500 to obtain K^+ concentrations measureable by atomic absorption spectroscopy. As a consequence of this, measurement of small cellular K^+ fluxes proved to be difficult. Thus, an experimental system was devised using a high cell density, a large metal addition and a K^+-free buffer (either 50 mM MES pH-adjusted with the addition of 10 mM NaOH, or 50 mM Tris/succinate buffer) whereby monitoring the sample filtrate any metal - K^+ exchange should be theoretically detectable at high cell metal loadings.

Using this system, several observations were made. On addition of washed cells to the K^+-free buffer, a small release of cell K^+ occurred and this is in accordance with the findings of Pena (1978). This may have been due to 'carry-over' of growth medium, however due to the effectiveness of the cell washing procedure this seems unlikely. For example, if the number of washes was increased to 6 with no K^+ detected in these washes, the same release still occurred suggesting that a small amount of K^+ left the cells when placed in K^+-free medium. In addition, it was seen that this K^+ released (or

Table 8.3.1. Mn^{2+} / H^+ exchange

	H^+ release μmol (g dry wt)$^{-1}$ min^{-1}
Metabolising cells	1.42 ± 0.11
Metabolising cells + 200 μM Mn^{2+}	1.99 ± 0.14
Difference	0.57 ± 0.12
Mn^{2+} uptake rate (μmol g^{-1} min^{-1})	0.23 ± 0.19
Mn^{2+}in / H^+out	1 : 2.5

Values given are the mean ± S.E. of 4 separate determinations

in fact any K^+ added during the incubation) was immediately and rapidly reinternalised indicating that the K^+ influx system is still operational under such conditions.

On the basis of these results and also due to the lack of any detectable K^+ release during incubation of cells with 200 μM Mn^{2+} or 5 μM Cu^{2+} in either of the K^+-free buffers, it was decided to examine for H^+ release during metal uptake. Owing to the toxic nature of Cu^{2+}, it proved impossible to obtain sufficiently high Cu^{2+} loadings in cells in order to detect concomitant monovalent cation release. However, using a Mn^{2+} addition of 200 μM, a proton efflux was observed (Table 8.3.1.). Metabolising cells released H^+ ions at a considerable rate in the presence of glucose, yet when 200 μM Mn^{2+} was also present, an additional proton release was readily detectable. This additional H^+ loss occurred at a rate 2.5 times greater than the Mn^{2+} influx rate. Hence a ratio of Mn^{2+}_{in} : K^+_{out} of 1 : 2.5 was obtained.

8.4. Discussion

Mn^{2+} efflux appeared to be an energy-dependent process. This observation was borne out in cells preloaded with either high or low Mn^{2+} concentrations and is in agreement with previous reports in which metal efflux was also blocked by the absence of glucose or the presence of metabolic inhibitors (Silver & Clark, 1971, Boutry *et al*, 1977). Passive diffusion of cations from the cells seems most unlikely as a mechanism of efflux. As no measureable diffusion of metals into cells occurred, the diffusion of metals out of cells would probably not occur either. When cells are suspended in

Mn^{2+}-free buffer plus glucose, any Mn^{2+} released from the cells is likely to be rapidly accumulated by either the Mn^{2+} specific uptake system or the low specificity Mg^{2+} transporter. If the rate of recapture of Mn^{2+} is greater than its rate of release, which seems to be the case here, then no overall Mn^{2+} loss would be detected. However, when 5 mM $MgCl_2$ is added, as in Figure 8.3.1., any Mn^{2+} released would be diluted by the Mg^{2+}, and the Mn^{2+} recapture rate would be greatly reduced as both Mn^{2+} and Mg^{2+} would compete for transport *via* the Mg^{2+} carrier (the Mn^{2+}-specific uptake system would be saturated at these high concentrations of released Mn^{2+}). This would lead to an overall cellular loss of Mn^{2+}. The effect of external divalent cations promoting egress of Mn^{2+} and Mg^{2+} has been previously reported in bacteria (Jasper & Silver, 1977, Silver & Jasper, 1977) and appears to be of a similar nature to these observations.

Efflux of ^{54}Mn from cells with comparatively low intracellular Mn^{2+} concentrations appears to be of the same type as seen in high Mn^{2+}-loaded cells. It is obvious from these experiments that the efflux rate is not constant but probably varies as a function of the cell Mn^{2+} content. Mn^{2+} efflux from high Mn^{2+}-loaded cells occurred at a rate many times greater than that observed in low Mn^{2+}-loaded cells. It seems unlikely that the 5-fold decrease in the concentration of divalent cations used to promote egress would account for this difference as saturation of even the low affinity Mg^{2+} transport system would occur at these millimolar concentrations and it would be operating at maximum capacity. Mn^{2+} egress in *B.subtilis* occurs at a constant rate throughout a regulatory cycle of intracellular Mn^{2+} levels during sporulation (Silver, 1978), however increasing the external Mn^{2+} concentration has the effect of

accelerating the Mg^{2+} efflux rate in *E.coli* (Silver & Clark, 1971). The important rate-controlling factor in these studies was the intracellular metal concentration. Saturation of Mg^{2+} egress from *E.coli* was never observed and means only that system-saturating levels of internal Mn^{2+} were not established. Thus it seems that in *C.utilis* a similar pattern of events to those described in Figure 8.1. may occur. Uptake of Mn^{2+} or Mg^{2+} leads to an elevated intracellular accumulation of these metals which may subsequently displace Mn^{2+} bound to cellular components such as polyphosphates (Borst-Pauwels, 1981). The displaced Mn^{2+} would then be free to be released from the cell *via* the efflux mechanism.

The identity of the efflux system is not clear. There are several possibilities however. Silver (1978) has stated that all cell types appear to have outwardly orientated Ca^{2+} transport systems and it is possible that other divalent cations may act as alternative substrates for this system. Thus, in the present study, Mn^{2+} may be released from cells *via* the Ca^{2+} efflux mechanism. Indeed, Theuvenet *et al* (1986) have suggested that Mn^{2+} and Sr^{2+} extrusion in *S.cerevisiae* occurs by way of the Ca^{2+} efflux system and ascribed the differential efflux of these cations to a greater affinity of Sr^{2+} for this system. If this was the case in *C.utilis*, then the relatively low rate of Mn^{2+} efflux observed could be explained by the low affinity of Mn^{2+} for the system as, to exert a physiological role, the transporter should have a high affinity for Ca^{2+} and much lower affinities for Mn^{2+} and Mg^{2+}. Alternatively, Nelson and Kennedy (1971) have considered it unlikely that influx of Mg^{2+} in *E.coli* is mediated by a system separate from that which catalyses efflux and have postulated that the same mechanism is responsible for transport in both directions. In this manner Mg^{2+}/Mg^{2+} exchange

could be performed by a transporter constantly shuttling backwards and forwards across the cytoplasmic membrane (Flatman, 1984). Low affinity substrates for the Mg^{2+} carrier such as Mn^{2+} or Co^{2+} could be similarly transported in either direction (Silver, 1978). In addition, the affinities of the solute for the influx and efflux transport sites may differ. Unfortunately, the available data from this and other efflux studies does not allow differentiation between the two alternative systems postulated and there is clearly scope for further studies to investigate this. It would be of interest to use inside-out yeast plasma-membrane vesicles to examine divalent cation uptake *via* this system.

As in a study of Mn^{2+} efflux in *L.plantarum* (Archibald & Duong, 1984), the total initial uptake velocity here was not greatly affected by the exit of Mn^{2+} in conditions of either high external Mn^{2+} or trace Mn^{2+}. In high Mn^{2+}-loaded cells, efflux of Mn^{2+} occurred at only 8 % of the V_{max} of Mn^{2+} uptake *via* the Mg^{2+} transporter whilst at low Mn^{2+} concentrations, the rate of Mn^{2+} release is less than 1 % of the V_{max} of the Mn^{2+}-specific uptake system. The real effect on the kinetic data is likely to be even less when it is considered that these values are for Mn^{2+} efflux following incubation in 100 μM Mn^{2+} or 10 nM ^{54}Mn over a period of 30 min after which the cellular levels (and consequent Mn^{2+} efflux) were greatly elevated. In contrast, the kinetic determinations were made during the initial few minutes of uptake when the total cell Mn^{2+} levels were low and loss of Mn^{2+} by way of the efflux system was likely to be minimal.

The lack of detectable Cu^{2+} efflux in this study is not an isolated observation. Zn^{2+} transport in a different strain of *C.utilis* was

apparently unidirectional with no efflux occurring from ^{65}Zn-loaded cells in the presence of glucose and 200 μM non-radioactive Zn^{2+} (Failla *et al*, 1976, Lawford *et al*, 1980). In Cu^{2+}-loaded cells of the fungus, *Dactylium dendroides*, Cu^{2+} rapidly bound to cell constituents and could not be released from the cells (Shatzman & Kosman, 1978) whilst in the yeast, *Candida maltosa*, no radioactive Co^{2+} efflux occurs when cells are incubated for 15 min in radioactive Co^{2+} then transferred to medium containing non-radioactive Co^{2+} (Belov *et al*, 1985). The great majority of the intracellular pool of Co^{2+} was found to be in the cell vacuoles or sequestered to Co^{2+}-binding proteins and thus unavailable for efflux. It was not determined whether other divalent cations could displace this bound fraction of Co^{2+}. No Mn^{2+} efflux was observed in *S.cerevisiae*; on addition of non-radioactive Mn^{2+} very little back-exchange of cellular ^{54}Mn occurred indicating irreversibility of uptake (Rothstein, 1958) and in the same species, Fuhrmann & Rothstein (1968) suggested that Zn^{2+}, Ni^{2+} and Co^{2+} were also taken into cells in a non-exchangeable form. Furthermore, Macara (1978) stated that it was unlikely that Cd^{2+} adaptation in *S.cerevisiae* involved an efflux mechanism as, after 1 h incubation in 5 mM $CdCl_2$, no release of ^{109}Cd was seen in ^{109}Cd-loaded cells.

Some metal ions, once they are covalently sequestered within macromolecules in biological systems, are difficult to replace with other competing metals (Wood & Wang, 1983) and it is not clear whether this explains the lack of efflux of Cu^{2+}, as compared to Mn^{2+}, in *C.utilis*. The Cu^{2+} ions may not be free in the cytoplasm but may be specifically or non-specifically bound to a Cu^{2+}-sequestering macromolecule. Cu^{2+}-binding proteins similar to mammalian Cu^{2+}-thioneins have been detected in yeasts (Naiki &

Yamagata, 1976) and if their affinity for Cu^{2+} is much greater than for other divalent cations, displacement of Cu^{2+} by these cations may not occur. Alternatively, if Cu^{2+} has no affinity for the efflux system present then no cellular release of Cu^{2+} would be expected.

Divalent cation - monovalent cation exchanges in order to maintain electroneutrality were apparent in this study of *C.utilis*. An experimental ratio, $Mn^{2+}_{in} : H^{+}_{out}$ of 1 : 2.5 indicates a stoichiometric exchange of 2 H^{+} released for each Mn^{2+} taken up by the cells. It is well established that acidification of the medium by metabolising yeast cells results from proton extrusion and release of acid organic products of metabolism (Theuvenet & Bindels, 1980). Hence it was necessary to subtract this H^{+} release from the total H^{+} loss observed on addition of Mn^{2+} in order to calculate the rate of H^{+} extrusion directly resulting from Mn^{2+} influx. Metal-proton exchanges to maintain cellular electroneutrality have been previously reported in microorganisms (Conway & Beary, 1958, Lichko *et al*, 1980, Wood, 1984a) although the majority of studies of metal uptake in yeasts have detected a concomitant K^{+} release (reviewed by Borst-Pauwels, 1981).

It is significant that in all these studies of K^{+} release coupled to metal influx no account appears to have been taken of subsequent reinternalisation of released K^{+} by cells. Under the experimental conditions imposed during these studies there is no reason to suppose that the monovalent cation uptake system is not operating as normal. Certainly, in this study any K^{+} ions released in response to Mn^{2+} uptake may have been taken back into cells *via* the K^{+} transport system and therefore may have been undetected. K^{+} uptake in *Neurospora crassa* and *S.cerevisiae* is coupled to H^{+} excretion

facilitated by a plasma-membrane ATPase (Goffeau & Slayman, 1981, Jennings, 1983). It seems possible therefore that any K^+ released in exchange for Mn^{2+} may be subsequently reaccumulated by this K^+/H^+ ATPase and exchanged for protons. Thus the overall effect would be a stoichiometric transfer of $2H^+ : 1Mn^{2+}$. In view of the fact that no K^+ release was detected when 10 mM Na^+ was added in an attempt to saturate the monovalent cation transporter and prevent subsequent K^+ influx, the latter explanation does not seem as plausible. It should be noted that the affinity of Na^+ for the monovalent cation carrier is some 32 times less than K^+ (Borst-Pauwels, 1981), however a Na^+ concentration of 10 mM should, in theory, easily suffice to block uptake of released K^+. Furthermore, the Mn^{2+} uptake rate was sufficiently high such that any K^+ loss should have been observed.

Thus it seems likely that in *C.utilis* a direct exchange of 2 protons occurs for each Mn^{2+} ion entering although the possibility of an indirect exchange by way of the K^+/H^+ transport system cannot at this stage be precluded. The presence of an antiport system has been suggested for Ca^{2+}/H^+ transport, however as a direct role of the plasma-membrane ATPase in divalent cation transport has been ruled out (Nieuwenhuis *et al*, 1981) and as H^+ extrusion in yeasts is *via* a membrane-bound ATPase (Goffeau & Slayman, 1981), it seems probable that metal influx and H^+ release occur at different sites. This view is supported by a study of Cd^{2+} transport in *A.pullulans*, for which an antiporter model did not apply for concomitant monovalent cation release, with Lineweaver-Burk plots of uptake and efflux indicating that they did not occur at the same cellular site (Mowll & Gadd, 1984).

This evidence that Mn^{2+} transport is linked with H^+ extrusion provides further evidence that an electrogenic release of protons may serve as the driving force of divalent cation uptake, as suggested in Chapter 5. Alternatively, as in other cell types, if K^+ loss is coupled to metal influx, then accumulation of cell K^+ may act as the store of a form of energy that can be used for uptake of divalent cations and other materials as postulated by other authors (Pena, 1978, Lichko *et al*, 1980). In either case, the movement of monovalent cations in yeasts appears to be intimately linked to the translocation of other ions including metals.

In conclusion, the work described in this chapter has demonstrated that a metabolism-dependent efflux of Mn^{2+} occurs in *C.utilis*, albeit to a small extent compared to the uptake of this cation, and that Mn^{2+} influx is balanced by an overall stoichiometric loss of protons from the cell. In contrast, Cu^{2+} could not be displaced from the cell under similar conditions and may be partitioned or tightly-bound within the cell. The interactions of these metals with the cellular transport systems will be further investigated in the following chapter when the effect of certain ions, including monovalent cations, is examined in the light of the data presented in this section.

9. EFFECT OF pH, POTASSIUM AND PHOSPHATE IONS ON DIVALENT CATION TRANSPORT

9.1. Introduction

In Chapter 8, the close relationship between the cellular movement of monovalent cations and divalent cation transport was emphasised. This chapter examines the effect of external concentrations of H^+ and K^+ cations and phosphate anions on divalent cation uptake. The presence of these ions in the medium can have a significant effect on transport and thus it is important to understand these interactions in order to draw conclusions based on comparative uptake studies.

The nutritional status of the medium is markedly affected by pH at a number of different levels. The availability of ions to microorganisms and hence their suitability for transport is determined by the solubility of ions and the dissociation of molecules which varies as a function of pH. Metal ions are most soluble in acid solutions, and at pH values near neutrality, formation of insoluble metal-anion complexes can occur. For example, Baldry and Dean (1980b) reported that the solubility of Cu^{2+} in MES buffer was around 1000 mg l^{-1} at a pH of 5, yet was only 8.2 mg l^{-1} at pH 7. This is reflected, to a certain extent, by the resultant growth under acid conditions; the growth of *Penicillium ochro-chloron* in high Cu^{2+} concentrations, measured by the increase in mycelial dry weight, was greatly diminished in the presence of 0.1 % HCl, H_2SO_4 or HNO_3 (Basu *et al*, 1955). This effect was thought to have been due to Cu^{2+} poisoning of the cells owing to the greater availability of the metal in acid solution. Thus the uptake of a metal must vary in accordance with its solubility; the more metal available in solution,

the greater the transport rate.

The thermodynamics of trace metal transport depend, amongst other factors, on the Δ pH (ie. the internal versus the external pH) (Wood, 1984a). The cytoplasm of microbial cells is very well buffered against pH changes and the internal pH varies only slightly as a result of environmental pH changes. However the cell pH is one of the factors regulating ion transport in yeasts and it is reported that the V_{max} of monovalent cation transport increases as the cell pH decreases and that divalent cation transport is also enhanced on decreasing the cell pH (Borst-Pauwels, 1981). A recent article by Slavik and Kotyk (1984) has shown that in weak buffers, the pH pattern throughout the yeast cell was not homogenous but decreased gradually towards the cell periphery to attain the pH value of the bulk medium. Thus, pH measurements between the cell as a whole and the bulk solution are unreliable and probably give a great overestimation of the actual Δ pH across the yeast cell membrane. Nevertheless, the Δ pH can provide an important driving force for membrane transport of ions.

In addition to those mentioned above, other factors such as the effect of increased H^+ concentrations on the cell surface potential, and competitive effects for negatively-charged transport sites, all play a role in determining the effect of pH on cation transport and will be discussed later. There is a wealth of literature detailing variations in cation uptake with increasing or decreasing pH as the following selected examples in yeasts and fungi illustrate.

pH profiles of Ni^{2+} uptake in *Neurospora crassa* clearly show an optimum uptake at pH 4.0 (Mohan *et al*, 1984) and in the same species,

Co^{2+} uptake increased linearly with pH within the pH range 3 to 6 (Venkateswerlu & Sastry, 1970). Beyond pH 6, precipitation of Co^{2+} as a hydroxide salt occurred. *C.utilis* cells accumulated 100 % of added 5 μM Ag^{+} in a phosphate buffer at pH 3 to 5, whilst at pH 6 to 8 only 60 % was accumulated as the remainder existed as an insoluble form of silver phosphate (Golubovich *et al*, 1976). Uptake of Zn^{2+}, also in *C.utilis*, increases with increasing solubility and effective Zn^{2+} concentration, as the pH decreases from 8 to 5 (Failla *et al*, 1976). The effect of pH on monovalent cation transport is rather more complex, exerting both competitive and non-competitive effects depending upon the buffer pH (Armstrong & Rothstein, 1964). Conway and Beary (1958) described the effect of H^{+} ions on Mg^{2+} uptake *via* the monovalent cation carrier with uptake reaching a plateau of maximum transport at pH 6.5 to 7.0 and virtually no uptake occurring below pH 5.0. The inhibitory effect on transport of very low pH values is highlighted in the case of acidophilic fungi. Generally most Cu^{2+} tolerant microbes are acidophiles in which Cu^{2+} resistance may be due to the ability of H^{+} ions to compete with Cu^{2+} ions rendering the cell impervious to Cu^{2+} (Okamoto *et al*, 1977). For example, *Scytalidium sp.* was capable of growth in 1 M $CuSO_4$ at pH 2 to 3, yet at a pH near neutrality was sensitive to just 40 μM Cu^{2+} (Starkey, 1973). Furthermore, Gadd and Griffiths (1980) concluded that variations of uptake with pH was, in part, the basis of Cu^{2+} tolerance in *Aureobasidium pullulans*. At pH 4.5, the Cu^{2+} tolerant strain took up less Cu^{2+} than a sensitive strain whilst a lowering of pH from this level decreased Cu^{2+} uptake in both strains.

A number of the effects on metal uptake due to elevated H^{+} concentrations will also occur in the presence of high external K^{+}. These would include the role of K^{+} in altering the negative potential

at the surface of yeast cells and the inhibitory effect at high K^+ concentrations on cation uptake due to competition for the transport sites. As amino-acid transport was tightly coupled to K^+ efflux in *Saccharomyces carlsbergensis*, increasing the K^+ concentration inhibited the uptake rate of glycine by around 80 %, K^+ behaving as a non-competitive inhibitor of the transport system (Eddy *et al*, 1970a). Fuhrmann and Rothstein (1968) showed that the uptake of Zn^{2+}, Ni^{2+} and Co^{2+} in *Saccharomyces cerevisiae* was inhibited in a non-competitive manner by an excess of K^+, whilst Norris and Kelly (1977) demonstrated that the addition of K^+ (or in fact Na^+) as a sulphate at equimolar concentrations to Co^{2+} or Cd^{2+} caused only a small inhibition of the divalent cation uptake rates. A reduction in the cell transport rates of Cu^{2+} and Ca^{2+} caused by high K^+ concentrations has been observed in *A.pullulans* (Gadd & Mowll, 1985) and in *S.cerevisiae* (Borbolla & Pena, 1980) repectively, where, in the latter study, 50 mM K^+ reduced Ca^{2+} uptake to half that observed in the absence of K^+.

In a review of inorganic ion nutrition in *S.cerevisiae*, Jones and Greenfield (1984), citing the data of several other authors, reported a stimulatory effect on Mn^{2+} uptake by K^+ concentrations upto 20 mM whilst at K^+ levels greater than this Mn^{2+} uptake was reduced and that at the optimum K^+ concentration required for growth, maximal Mg^{2+} uptake occurred. Rothstein *et al* (1958) also observed this stimulation of Mn^{2+} uptake at 3 to 8 mM K^+, with 100 mM K^+ strongly inhibiting uptake. They attributed the inhibitory effect of K^+ to an excess of K^+ accumulated within the cell, whereas the stimulatory effect was ascribed to being a result of increased phosphate influx, as the uptake of Mn^{2+} also appeared to be linked to phosphate transport. A role of K^+ in glycolysis activation and in stimulation

of glucose transport in *S.carlsbergensis* has been noted (Lichko *et al*, 1980), however no concomitant elevation of Mn^{2+} influx was observed. Indeed, in the same study Mn^{2+} uptake was impeded to a small extent even by 1 mM K^+. In contrast to these reports, a Mg^{2+} transporter which was highly sensitive to K^+ has been described in *S.cerevisiae* (Conway & Beary, 1958). Uptake of Mg^{2+} from 200 mM magnesium acetate was inhibited 50 % by just 0.58 mM K^+ indicating that the relative affinities of K^+ and Mg^{2+} for the system were 700 : 1. A similar degree of inhibition was seen using the cations Rb^+ or Cs^+. It appears that Mg^{2+} influx in this case was *via* the monovalent cation carrier and not the divalent cation uptake system.

Early work in Rothstein's laboratory outined the importance of the presence of phosphate during uptake of Mg^{2+} or Mn^{2+} in yeasts (Rothstein *et al*, 1958). If phosphate was absent, no cellular uptake of Mn^{2+} occurred and only energy-independent surface binding was observed. However when phosphate was present with cells which had been pre-exposed to K^+ and glucose, a remarkable stimulation of Mn^{2+} uptake was evident. It was concluded that the uptake rather than the presence of phosphate was the important factor in the stimulation of Mn^{2+} influx. Subsequent work by Jennings *et al* (1958) led to the postulation of a model for divalent cation uptake whereby synthesis of the divalent cation carrier involved a phosphorylation step which was linked to the transport of phosphate. This model is reproduced in Figure 9.1. The carrier X is synthesised during glycolysis and facilitates the translocation of phosphate. The phosphorylated form of the carrier XP is in part converted to YP which can then facilitate transport of Mn^{2+}.

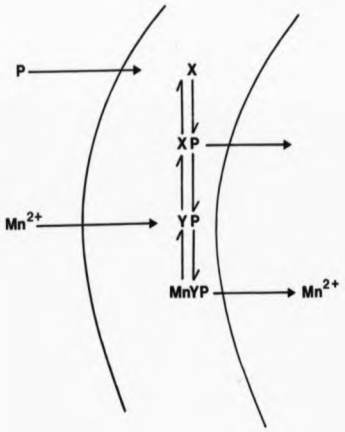


Figure 9.1. Model for transport of Mn^{2+} and phosphate (after Jennings *et al*, 1958)

More recently, it was observed in *S.cerevisiae* that uptake of the cations, Mg^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+} and Ca^{2+}, was increased 5 - 20-fold when starved cells were pretreated with K^{+}, glucose and phosphate (Fuhrmann, 1974a). However, the pretreatment increased the cell ATP content 5-fold, and the initial rate of divalent cation uptake was directly proportional to the cell ATP concentration. Also, during uptake, the ATP was partially used up suggesting that ATP was necessary for divalent cation uptake. This point was also noted by Okorokov *et al* (1979) who showed that the ability of *S.cerevisiae* to take up Mn^{2+} was related to the ATP content during exponential phase and that polyphosphates could probably act as endogenous energy reserves for Mn^{2+} transport with 3 Mn^{2+} ions translocated for each polyphosphate bond broken. However if glucose was present, this did not occur indicating that polyphosphates only provided an energy supply if no exogenous source was available.

Other data include a 3-fold stimulation of Cd^{2+} uptake in phosphate buffer as opposed to a PIPES buffer observed in *S.cerevisiae* (Norris & Kelly ,1979). This increase was independent of the presence of glucose and the possibility of precipitation of cadmium phosphate at the cell surface or onto filters was not excluded. In general, phosphate has little effect on bacterial cation transport systems, although the presence of inorganic phosphate stimulated Ca^{2+} uptake severalfold in vesicles of *Bacillus subtilis* (de Vrij *et al*, 1985). This has also been seen in mitochondria and in the sarcoplasmic reticulum and has been attributed to the formation of insoluble calcium phosphate complexes inside the vesicles which would effectively alter the Ca^{2+} concentration gradient.

Hence the importance of K^+, H^+ and phosphate in the suspending buffer during uptake experiments can be seen. This section aims to provide knowledge of the effects on transport which may be caused by these important ions and to know whether the uptake systems studied can be further characterised on the basis of their response to H^+, K^+ or phosphate.

9.2. Materials and methods

To investigate the effect of varying pH on metal uptake, a pH-stat (Pye-Unicam) was employed to maintain the pH at a preset level. This was achieved by the addition of 0.1 M KOH as a titrant into a buffer-free solution of 50 mM glucose. Uptake experiments were carried out at 30 ^{o}C in a 600 ml glass vessel with temperature controlled water-jacket using a total cell suspension volume of 200 ml. Agitation was provided by a magnetically-coupled stirrer bar. Samples were removed following 20 min incubation with the metal salt which was added at a final concentration of 10 nM ^{54}Mn, 50 µM non-radioactive Mn^{2+} or 5 µM Cu^{2+}.

All other experiments described in this chapter were carried out using shake-flasks and a buffer containing 50 mM MES and 50 mM glucose (pH 5.5) as previously outlined in Chapter 3. K^+ concentrations were altered by the addition of KOH. Phosphate was added in the form of KH_2PO_4. To measure the effect of K^+, KOH was added 1 min prior to the addition of a metal whilst the effect of phosphate was determined following the addition of metal to a suspension of cells to which 2 mM KH_2PO_4 had been added 30 min previously. Mn^{2+}, at 10 µM, or Cu^{2+} at 5 µM was added and cells were

incubated with the metal for 15 min prior to sampling.

9.3. Results

9.3.1. Effect of pH on uptake

To account for the possibility that addition of low concentrations of K^+, in the form of KOH used as the titrant, affected uptake of the metals, parallel experiments were carried out using Tris/succinate buffer at the same pH values as used with the autotitrator set-up. Identical results were seen in both experimental conditions suggesting that the titrant had no effect on subsequent uptake. The selection of KOH as a titrant in preference to NaOH was unimportant. Both monovalent cations are likely to exert similar effects, if any, at the low concentrations at which they were added during these experiments.

Figure 9.3.1. describes uptake from 50 μM Mn^{2+} in the pH range 2 to 6. Uptake appeared to be strongly dependent upon pH with a plateau of maximum uptake occurring at pH 5.5 to 6.0. At pH values 7.0 and 8.0, a massive increase of cell Mn^{2+} was noted as all the Mn^{2+} added appeared to be associated with the cells. This was presumably due to precipitation of Mn^{2+} onto the cell surfaces at these high pH values. Indeed, visible precipitation of Mn^{2+} occurs when $MnCl_2$ is added to this buffer system and the pH raised to around neutrality.

Figure 9.3.2. shows the effect of pH on uptake from 10 nM ^{54}Mn *via* the Mn^{2+}-specific transport system. Uptake was highly dependent upon the pH of the suspending medium. As the external H^+ concentration was increased, ^{54}Mn uptake decreased sharply with only 5.1 % of the

uptake seen at pH 5.5 occurring at pH 3.0. No plateau of maximum uptake was observed although at pH 7.0 and above a large increase to over 15 nmol ^{54}Mn (g dry wt)$^{-1}$ cells was recorded. It is probable that, in this case also, insoluble complexes of manganese hydroxide precipitate onto the cell surface at high pH.

A somewhat different pH profile was seen for Cu^{2+} uptake (Fig. 9.3.3.). Optimum uptake occurred at a pH range 5.0 to 5.5. Outside this range, uptake decreased sharply with around 40 % of the maximum uptake occurring at pH values of 3.5 and 7.0.

9.3.2. Effect of potassium on uptake

The effect of increasing K^+ concentrations on the uptake of Mn^{2+} from 10 µM Mn^{2+} is shown in Figure 9.3.4. At K^+ concentrations greater than 10 µM, the total Mn^{2+} uptake after 20 min was reduced by 45 %. Increasing the K^+ concentration to 150 mM appeared to have no greater inhibitory effect than that observed at 50 mM K^+. A slightly different effect is seen for Cu^{2+} uptake (Fig. 9.3.5.) where Cu^{2+} uptake from 5 µM Cu^{2+} decreased with increasing K^+ levels upto 150 mM.

9.3.3. Effect of phosphate on uptake

The effect that the presence of phosphate has on uptake is shown in Table 9.3.1. Uptake of Mn^{2+} from 10 µM Mn^{2+} was markedly stimulated some 3.5-fold by the presence of 2 mM phosphate whereas Cu^{2+} uptake remained unaffected. To take account of the possibility of a metal-phosphate precipitate being produced, the additions of metal ions and phosphate were made in the absence of cells. The resulting solutions were vacuum filtered through 0.22 µm membrane filters to remove any precipitate formed. Digestion and subsequent analysis of

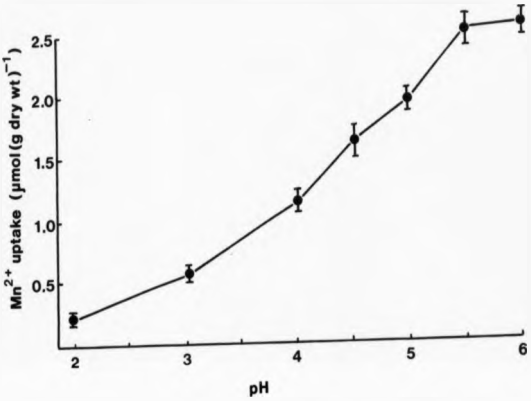


Figure 9.3.1. Effect of pH on Mn^{2+} uptake from 50 μM Mn^{2+} following 20 min exposure to the metal. Values represent the mean ± SE of 3 determinations.

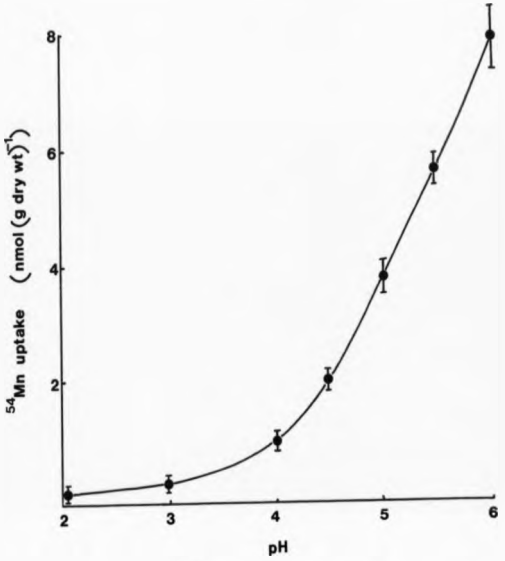


Figure 9.3.2. Effect of pH on ^{54}Mn uptake from 10 nM ^{54}Mn following 20 min exposure to the metal. Values represent the mean ± SE of 3 determinations.

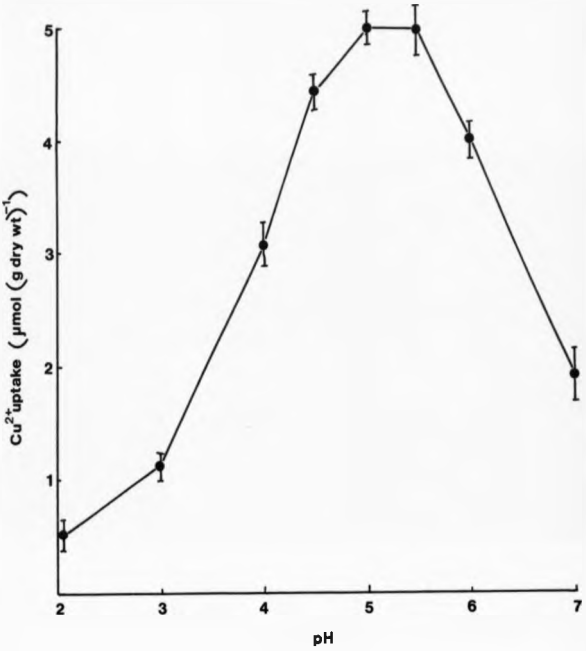


Figure 9.3.3. Effect of pH on Cu^{2+} uptake from 5 µM Cu^{2+} following 20 min exposure to the metal. Values represent the mean ± SE of 3 determinations.

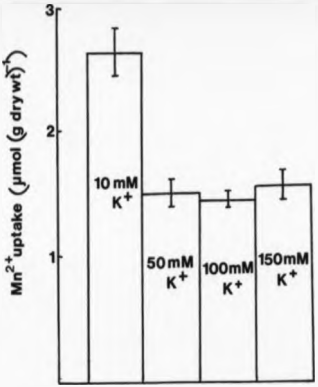


Figure 9.3.4. Effect of K^+ on Mn^{2+} uptake from 10 µM Mn^{2+} following 20 min exposure to Mn^{2+}. Values represent the mean ± SE of 3 determinations.

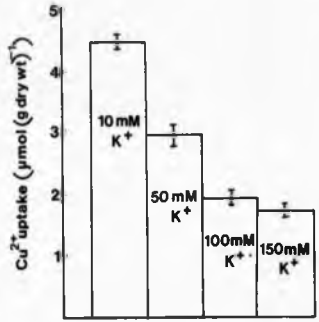


Figure 9.3.5. Effect of K^+ on Cu^{2+} uptake from 5 µM Cu^{2+} following 20 min exposure to Cu^{2+}. Values represent the mean ± SE of 3 determinations.

Table 9.3.1. Effect of phosphate on Cu^{2+} and Mn^{2+} uptake

Metal	Metal uptake [μmol(g dry wt)$^{-1}$]
Cu^{2+}	3.21 ± 0.08
Cu^{2+} + phosphate	2.88 ± 0.07
Mn^{2+}	1.74 ± 0.11
Mn^{2+} + phosphate	5.99 ± 0.08

Metals were added to a cell suspension to which 2 mM phosphate, in the form KH_2PO_4, was added 30 min previously. The final Cu^{2+} and Mn^{2+} concentrations were 5 μM and 10 μM respectively. Uptake was measured after 15 min incubation and the values represent the mean ± standard error of 3 experiments.

the filters revealed no metals present, suggesting that insoluble phosphate salts were not formed.

9.3. Discussion

The transport of metals by way of each of the uptake systems studied here is highly pH dependent. This finding is compatible with the observations of other authors. Mn^{2+} uptake in *S.cerevisiae* was maximal at pH 5 (Okorokov *et al*, 1979) and optimal uptake of Mn^{2+} in *Lactobacillus plantarum* was observed at pH 5 in a complex tryptone-mineral salts medium (Archibald & Duong, 1984). Similarly, pH profiles for Cu^{2+} uptake which correspond closely to the findings of this study have been reported in the literature. Cu^{2+} transport in the yeast, *Debaryomyces hansenii* was greatest at pH 5.0 and markedly reduced at pH values greater or less than 5.0 (Wakatsuki *et al*, 1979, 1985) whilst Gadd and Mowll (1985) reported maximal Cu^{2+} uptake in *A.pullulans* at pH 6.5 with only 20 % of this uptake occurring at pH 2.5. The likelihood of metal-hydroxide complexes forming at high pH values, precipitating onto cell surfaces and thus blocking transport of metal into the cells has been emphasised by Baldry and Dean (1980b). At the pH at which all kinetic and other uptake experiments were carried out in this study, namely 5.5, the uptake of both Mn^{2+} and Cu^{2+} is at an optimum. This pH value, which is also the lower end of the useful buffering range of the MES buffer used, was carefully preselected in the light of similar data reported by other authors. Nevertheless, the need to monitor carefully the pH with respect to metal uptake experiments is underlined here.

The reason for the depression of metal uptake at low pH is not completely clear. Undoubtedly, effects resulting from direct competition between protons and metal ions for the negatively-charged transport sites must play an important role. The effect on the surface potential should also be considered. As the H^+ ion concentration is decreased, the surface potential becomes more negative and the concentration of divalent cations immediately adjacent to the cell membrane increases, resulting in elevated uptake (Borst-Pauwels, 1981). Roomans *et al* (1979) have suggested that the inhibition of Ca^{2+} and Sr^{2+} uptake at low pH must be a result of the reduction of the surface potential.

The effect of medium pH can be further complicated by the fact that the cell pH may also affect metal transport (Borst-Pauwels, 1981). As mentioned in Chapter 5, the proton gradient across a cell membrane can provide an important driving force for solute transport. Indeed, the previous chapter demonstrated how Mn^{2+} uptake *via* the non-specific Mg^{2+} transport system was intimately linked with proton release. Hence stimulation of transport should occur if the external H^+ concentration (and thus the proton gradient) is rapidly raised. This has been observed for proton-coupled amino acid uptake in *S.carlsbergensis* where the initial rate of glycine influx increased 5-fold when the pH was lowered from 7 to 4.5 (Eddy *et al*, 1970a). If the medium pH of a buffered suspension of cells is altered, then the cell pH gradually changes to a value nearer to that of the medium, with equilibrium being reached after 15 to 20 min in yeast cells (Slavik & Kotyk, 1984). During the present series of experiments, cells were pre-incubated in the medium at a determined pH for at least 30 min prior to metal addition. Thus the membrane proton gradient during metal uptake would not differ greatly with changes in

the external pH and any slight uptake stimulation due to an increased H^+ gradient would be masked by the much greater inhibitory effects described above. A pH effect was described by Roomans *et al* (1979) where, as the cell interior became more acidic, Sr^{2+} uptake in *S.cerevisiae* was stimulated due to increased H^+-pump activity. However this effect was of minor significance when compared to the effect of a decreased surface potential on Sr^{2+} influx.

One further explanation for the observed response to pH by cell metal transport systems is that it may be due to an indirect effect such as modification of ATPase activity. It is interesting to note that the pH optima for plasma membrane ATPase systems closely parallel those of metal uptake systems. The pH range at which maximum plasma membrane ATPase activity generally occurs in yeasts and fungi has been reported to be 5.0 to 7.5 (Goffeau & Slayman, 1981, Jennings, 1983). If metal transport was obligatorily coupled to either K^+ or H^+ release and the ATPases involved in these exchanges were inhibited by low pH, which seems likely, then metal uptake would be similarly inhibited by acid conditions.

The presence of high concentrations of K^+ seriously impeded uptake of both Mn^{2+} and Cu^{2+} in *C.utilis*. Again, the arguments which were applied in the discussion of the role of H^+ ions in cell transport are valid. In all probability K^+ will have a very low affinity for the divalent cation uptake systems and thus inhibition of metal uptake would only be observed at high K^+ concentrations. The predicted effect of K^+ on the surface potential, and hence uptake of metals, has already been described in Chapter 7 and cannot be precluded. Okorokov *et al* (1979) noted that the ability of *S.cerevisiae* to take up Mn^{2+} was partly related to the cellular K^+

concentration at all stages of growth and this may be the case in *C.utilis*.

If the influx of a divalent cation is relatively tightly linked with K^+ efflux, inhibition of the divalent cation uptake system should be expected with increasing exogenous K^+ concentrations (Lichko *et al*, 1980). This has been demonstrated in *A.pullulans* where the internal and external K^+ concentrations exerted a strong influence on Cu^{2+} uptake and according to these values, the rate of Cu^{2+} influx could be controlled (Gadd & Mowll, 1985). In the same study, Cu^{2+} transport, which is linked with a stoichiometric release of K^+, was negligible under conditions where the K^+ concentration gradient inhibited K^+ efflux. This was seen when the external K^+ concentration was equal to, or greater than, the intracellular K^+ concentration. As the extracellular K^+ level approaches that of the intracellular K^+, the membrane potential will collapse and processes dependent upon this potential will be halted (Harold *et al*, 1974). Hence high external K^+ will lead to greatly increased cellular K^+ uptake, ultimately leading to depolarisation of the cell membrane. *C.utilis* cells used in this series of experiments and grown in the media descibed in Chapter 2, contained internal K^+ concentrations measured to be around 150 mM K^+. Therefore the suggestion that uptake may be linked to the membrane potential may be partly true as, in the case for Cu^{2+}, uptake was markedly reduced by 150 mM exogenous K^+. However, over 50 % of Mn^{2+} uptake still occurred under these conditions, providing further evidence to that described in Chapter 5 that energy-coupling to Cu^{2+} and Mn^{2+} transport differs somewhat.

As postulated in the previous chapter, if Mn^{2+} influx in *C.utilis* is balanced by ATPase proton release, rather than by K^+ loss followed by

K^+ / H^+ exchange, then the uptake system would be most sensitive to external H^+ concentrations as opposed to external K^+. This appears to be the case for Mn^{2+} uptake *via* the Mg^{2+} transport system in *C.utilis*. It seems likely that the inhibitory action of high exogenous K^+ levels is due to combination of effects resulting from changes in membrane potential, surface potential and transport site competition. It is not possible from this study to ascertain the exact role of each of the aforementioned factors.

The different responses to phosphate seen in Mn^{2+} and Cu^{2+} uptake is further proof that transport of these metals is catalysed by two distinct systems. No stimulation of Cu^{2+} uptake is seen in the presence of phosphate although this is not an isolated observation. Preincubation of *S.cerevisiae* cells with phosphate and K^+ did not increase Ca^{2+} uptake (Pena, 1978, Borbolla & Pena, 1980). In *C.utilis*, neither the pattern of Zn^{2+} uptake *via* a Zn^{2+}-specific carrier nor the total Zn^{2+} accumulated during the growth cycle were affected by the presence of 1.0, 10 or 100 mM KH_2PO_4 (Failla & Weinberg, 1977). The intracellular ortho- and polyphosphate levels were unchanged following growth in all three concentrations of phosphate and the authors also noted that in the bacteria, *Serratia marcescens* and *Pseudomonas aeruginosa*, neither Zn^{2+} nor Fe^{2+} uptake was affected by phosphate in the medium. In addition, Mn^{2+}-specific transport in bacteria has no requirement for phosphate and is not influenced by this anion (Silver & Jasper, 1977).

The observed stimulation of Mn^{2+} uptake *via* the non-specific Mg^{2+} carrier is in accordance with other reports of Mn^{2+} and Mg^{2+} uptake in *S.cerevisiae* (Rothstein *et al*, 1958, Fuhrmann, 1974a). It was not possible in this study to ascertain whether this stimulatory effect

was due to increased cell ATP content or was related to the formation of a phosphorylated product involved in divalent cation translocation, as described earlier. However, in contrast to the findings of Rothstein *et al* (1958), it was noticed that *C.utilis* cells were capable of taking up Mn^{2+} in the absence of phosphate, in cells which had not been pretreated with phoshate and also in starved cells to which glucose had been added.

Stimulation of Sr^{2+} uptake by phosphate in *S.cerevisiae* does not require the presence of extracellular phosphate, but occurs following phosphate uptake (Roomans *et al*, 1979), thus the possibility of phosphate-divalent cation cotransport is unlikely. It was speculated that either intracellular phosphate or a phosphorylated compound combined with the translocation mechanism to increase its activity. It seems equally unlikely that phosphate stimulation of Mn^{2+} uptake is due to an increase in the electrochemical potential difference across the cell membrane. The membrane potential is similar in both phosphate-rich and phosphate-deficient cells and, whilst there is a slight increase in the H^+ gradient across the membrane resulting from proton-phosphate symport and consequent increased H^+-pump activity (Roomans & Borst-Pauwels, 1977), this accounts for only a small fraction of the observed stimulation (Roomans *et al*, 1979). Thus, whilst the effect of phosphate on the non-specific divalent cation transporter is now well described in yeasts, the interactions of phosphate with this system at a molecular level remain unclear.

It has been possible in this chapter to further characterise the divalent cation transport systems in *C.utilis* in terms of the effects of H^+, K^+ and phosphate on them. For example, Cu^{2+}-specific uptake is strongly dependent upon both pH and K^+ ions yet is unaffected by

phosphate. This has also allowed, in conjunction with the data presented in Chapter 8, a further indication of the ways in which transport is coupled to an energy source and how elecroneutrality of the cell is maintained during divalent cation uptake. The strong influence on metal uptake systems of environmental factors such as pH and the presence of other ionic groups can be appreciated in the light of these results. Data of this nature are useful in that they will enable subsequent investigators to examine these transport systems under optimal operating conditions.

10. THE REGULATION OF TRANSPORT AND CELLULAR HOMEOSTASIS OF DIVALENT CATIONS

10.1. Introduction

An important feature of cellular metabolism is the ability to maintain homeostasis of inorganic ion concentrations in the cytosol. This is necessary under conditions of high external ion levels when toxic amounts of metals may be accumulated or during low nutrient stress when a paucity of essential cations in the environment may result in growth limitation. In addition, the rates and directions of many metabolic processes appear to depend on the concentrations of certain inorganic ions and thus control of these ions may well provide a means for metabolic regulation (Okorokov *et al*, 1980). There are a number of strategies by which microorganisms may regulate cellular metal ions and whilst several of these may have been invoked as mechanisms of resistance in metal-adapted cells, there is evidence that most, if not all, cells are capable of regulating their cytosolic ion levels.

Compartmentation of ions in the cell vacuoles has been thought to be effected by tonoplast transport systems. Cytochemical analysis and differential extraction of *Saccharomyces carlsbergensis* cells revealed predominant accumulation of K^+ and Mg^{2+} in vacuoles and the occurrence of concentration gradients of these ions across the tonoplast (Okorokov *et al*, 1977, 1980). It was assumed from this data that the tonoplast had a transport system for divalent cations, thereby regulating their concentration in the cytosol. A similar system was observed in intact cells of *Saccharomyces cerevisiae* where cytoplasmic Ca^{2+} homeostasis was maintained by a vacuolar Ca^{2+}

transport system (Eilam *et al*, 1985a, 1985b). As mentioned in Chapter 8, energy-driven metal efflux systems may operate in yeasts to reduce the internal concentrations of divalent cations such as Ca^{2+} and Sr^{2+} [see, for example, Theuvenet *et al* (1986)] and a further controlling factor may be the presence of metal-binding proteins (Failla & Weinberg, 1977) and polyphosphates (Okorokov *et al*, 1975). There are indications that formation and breakdown of intracellular polymeric magnesium orthophosphate is one of the ways in which the level of free cellular Mg^{2+} is regulated in filamentous fungi (Jasper & Silver, 1977). In *Candida maltosa*, transport of absorbed Co^{2+} into vacuoles and subsequent sequestration to Co^{2+}-binding protein has been proposed as a mechanism of Co^{2+} detoxification (Belov *et al*, 1985).

While many studies of metal ion transport in yeasts have been reported (Borst-Pauwels, 1981), few have considered the control of these transport systems. Such regulation may markedly affect cellular metal levels, particularly during exposure to toxic external concentrations of metal ions. Indeed, Ni^{2+} resistance in a Ni^{2+}-resistant strain of *Neurospora crassa* was specifically due to reduced activity of the Ni^{2+} uptake system (Mohan *et al*, 1984) whilst a Cu^{2+}-resistant mutant of *S.cerevisiae* took up less Cu^{2+} than the wild type, this being due to a change in the membrane transport properties (Gadd *et al*, 1984a).

Studies of Mn^{2+} uptake in bacteria (Eisenstadt *et al*, 1973, Archibald & Duong, 1984) and Mg^{2+} uptake (Nelson & Kennedy, 1972) have demonstrated repression of transport following growth in media containing high concentrations of Mn^{2+} and Mg^{2+} respectively. In these studies, Mn^{2+} uptake in Mn^{2+}-starved cells was subject to a

negative-feedback regulatory mechanism functioning 1 min after exposure to Mn^{2+} and was independent of protein synthesis (Archibald & Duong, 1984). Mutants of *Escherichia coli*, resistant to Co^{2+} and deficient in the non-specific divalent cation transporter, were shown to take up Mg^{2+} *via* a specific Mg^{2+} carrier some 50 times more rapidly when the cells were transferred from a high Mg^{2+} medium to a low Mg^{2+} medium (Nelson & Kennedy, 1972). Cells of *Candida utilis* grown in continuous culture under conditions of Zn^{2+} limitation, possessed the capacity to hyperaccumulate Zn^{2+} far in excess of the level attained in Zn^{2+}-supplemented medium (Lawford *et al*, 1980) indicating derepression of the uptake mechanism under these conditions. It was not clear whether Zn^{2+} homeostasis in *C.utilis* was achieved through control of the transport system *per se* or through the regulation of synthesis and breakdown of cytoplasmic Zn^{2+} sequestering macromolecules. One further example of divalent cation transport regulation was described in a filamentous fungus by Failla and Niehaus (1986). Zn^{2+} uptake in *Aspergillus parasiticus* varied with the Zn^{2+} content of the medium, the uptake rate at 30 h growth was inversely proportional to the mycelial Zn^{2+} content.

Whilst a number of other authors have found it impossible to create Mn^{2+}-deficient conditions as cells are capable of growth in media containing Mn^{2+} present as trace impurities of medium constituents (Silver, 1978), it is however possible to prepare Cu^{2+}-limiting growth medium by means of a selective chemical extraction procedure (Giorgio *et al*, 1963, Downie & Garland, 1973). This technique has been used to prepare Cu^{2+}-limited cultures of *C.utilis* in which the cell yield decreased with increasing Cu^{2+}-limitation and the cellular content of the Cu^{2+} containing cytochrome oxidase and cytochrome aa_3 was specifically reduced (Giorgio *et al*, 1963, Wohlrab & Jacobs,

1967, Light, 1972).

Continuous culture of microorganisms provides an excellent experimental system for studying the effects of growth limitation by various essential nutrients. There are several drawbacks to the use of batch culture to study metal ion limitation. Metals may be inadvertantly added to the medium through cell inocula or other contamination. Cultures may often require many cell doublings in metal-deficient media prior to the onset of growth limitation. Batch culture is considerably inferior to continuous in providing a reliable and reproducible supply of cells (Light & Garland, 1971). Indeed, van Uden (1971) has stated that, with *C.utilis* in batch culture, exponential growth may become unbalanced even in a constant environment and the specific growth rate may vary dependent upon unknown factors. However, steady states can be easily attained in continuous culture where accurate and repeatable changes in growth conditions leading to alterations in cell function can be readily monitored.

In addition to investigating the responses of cellular transport systems to changing external metal concentrations, it is also of interest to examine the total metal content of cells growing in batch culture. The reasons for this are two-fold: to monitor the metal transport systems and their regulation as the physiological state of the cells alters during the growth curve, and to examine for fluctuations in cellular metal content throughout the growth curve, as detailed in a number of reports which have been summarised below.

The cell Cu^{2+} content of *E.coli* in batch culture increased rapidly towards the end of the exponential growth phase, passed through a

sharp maximum in the deceleration phase and decreased in the stationary phase to a value one-quarter of the maximal Cu^{2+} content (Baldry & Dean, 1980a). Cd^{2+} uptake in a *Citrobacter* species increased sharply during the mid- exponential phase and declined in response to a fall in pH (Macaskie & Dean, 1984). Similar observations have been noted in yeasts and fungi. Zn^{2+} accumulation *via* a Zn^{2+}-specific carrier in *C.utilis* in batch culture was cyclic, uptake only occurring during the lag and late-exponential phases (Failla & Weinberg, 1977). Thus in this yeast, the Zn^{2+} content altered dramatically at different times in the growth cycle. The Mn^{2+} content of *S.cerevisiae* was greatest during the exponential phase (Okorokov *et al*, 1979) whilst Cu^{2+} uptake in *Geotrichum candidum* occurred during lag and mid- to late-exponential phases with minimal uptake in between (Quinn *et al*, 1981). Finally, the rate of Zn^{2+} uptake in *A.parasiticus* increased throughout the exponential phase to reach a maximum at the end of this phase (Failla & Niehaus, 1986) and in *Penicillium spinulosum*, Cu^{2+} accumulation was maximal during the lag phase (Townsley & Ross, 1985).

A number of complications can arise however when studies are made of growing cells. Metal transport processes are masked by the growth kinetics of the organism as the cells continuously increase in number, rendering it difficult to perform flux measurements on dynamic systems. Interference and complexation by medium components must also be considered and it is for these reasons that all previous uptake experiments in this study used have employed the use of washed, buffered suspensions of non-growing organisms to allow maximal uptake and allow comparison. This point has been emphasised by Townsley and Ross (1985) who stated that data obtained for resting mycelial suspensions to facilitate kinetic studies does not

necessarily provide an accurate indication of metal uptake in actively growing cells.

In this chapter, by taking advantage of the benefits gained from continuous culturing techniques, the regulation of the metal transport systems outlined in previous chapters is examined. In addition, the cellular Mn^{2+} content of *C.utilis* during growth in batch culture is studied with the aim of providing a clearer view of Mn^{2+} homeostasis under physiological conditions.

10.2. Materials and methods

10.2.1. Growth media

The basic culture medium contained the following components (g l^{-1}): sodium glycerophosphate, 1.0; $MgSO_4.7H_2O$, 1.0; $(NH_4)_2SO_4$, 3.0; $CaCl_2.6H_2O$, 0.25; KCl, 0.5; glucose, 10; inositol, 0.01; calcium pantothenate, 0.002; D-biotin, 0.0001; $FeSO_4.7H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.00175; $MnSO_4.4H_2O$, 0.0001; $CuSO_4.5H_2O$, 0.0001. The following adjustments were made to vary the medium composition: for low-Mn^{2+} medium, $MnSO_4.4H_2O$ was omitted (since a number of authors have attempted unsuccessfully to create truly Mn^{2+}-deficient conditions, Mn^{2+} was simply excluded from the medium, hence this medium was not Mn^{2+}-limiting, yet contained less Mn^{2+} than the basic medium); for high-Mn^{2+} medium, $MnSO_4.4H_2O$ was omitted and $MnCl_2$ added to a final concentration of 100 μM; for Cu^{2+}-limiting medium, the medium was subjected to the modified Cu^{2+} extraction procedure using zinc dibenzyldithiocarbamate in tetrachloromethane as described by Downie and Garland (1973); for high-Cu^{2+} medium, Cu^{2+} extracted medium was amended with $CuCl_2$ to a final concentration of 25 μM.

To select a suitable Mg^{2+} concentration for Mg^{2+} growth limitation experiments, a series of batch cultures were grown in the basic continuous culture medium containing 50 mM MES buffer and with varying Mg^{2+} concentrations as stated. These cultures were grown in 100 ml medium and harvested following 24 h growth. For low-Mg^{2+} medium, the basic culture medium was used with the omission of $MgSO_4.7H_2O$ and the addition of $MgCl_2$ to a final concentration of 25 μM.

For experiments measuring Mn^{2+} accumulation during batch culture, the synthetic medium of Failla *et al* (1976) was used [consisting of (mM): glucose, 16.5; NH_4Cl, 18.7; K_2HPO_4, 1.0; sodium citrate, 5.0; biotin, 0.004; piperazine-N-N-bis (2-ethane sulphonate) buffer, 5.6] supplemented with trace elements as follows (g l^{-1}): $MgSO_4.7H_2O$, 0.74; $CaCl_2.6H_2O$, 0.25; $FeSO_4.7H_2O$, 0.005; $ZnSO_4.7H_2O$, 0.00175; $CuSO_4.5H_2O$, 0.0001. The pH was adjusted to 6.0 by the addition of a saturated solution of KOH.

10.2.2. Continuous culture

A bench-top CC1500 chemostat (L.H. Engineering, Stoke Poges, U.K.) with a working volume of 1500 ml was used. Temperature was maintained at 30 ^{o}C. The air-flow rate was 2.5 l h^{-1}. pH was automatically maintained at 5.5 $\pm$ 0.1 using 2 M KOH as a titrant. Medium input was adjusted to give a dilution rate of 0.15 h^{-1}.

The glucose content of the medium outflow was determined using a spectrophotometric technique based on the conversion of glucose to glucose-6-P by ATP in the presence of hexokinase, coupled with the subsequent reduction of NADP to NADPH (Sigma reagent kit No. 15UV). As NADPH absorbs strongly at 340 nm whereas NADP does not, the

reaction can be followed by monitoring the change in absorbance at 340 nm. The NADPH produced is directly proportional to the glucose concentration.

At regular intervals samples of spent medium were taken, the cells removed by filtration and 0.02 ml filtrate added to 3 ml assay solution at 25 ^{o}C in a cuvette. Following thorough mixing and incubation for 5 min, during which the conversion of NADP to NADPH is completed, the absorbance at 340 nm was measured. The glucose concentration was calculated by subtracting the initial from the final absorbance reading and multiplying by a conversion factor (4400).

10.2.3. Batch culture

For experiments measuring Mn^{2+} accumulation in batch culture, medium (400 ml) in a 1 l shake-flask was inoculated with 0.1 ml of a 24 h starter culture; subsequent growth was at 30 ^{o}C on an orbital shaker (200 cycles min^{-1}). ^{54}Mn was added at an initial concentration of 1 nM and cell samples (5 ml) regularly removed throughout the growth curve for analysis. In the case of non-radioactive Mn^{2+} experiments, $MnCl_2$ was added as stated and cultures harvested at mid-exponential phase.

10.2.4. Metal ion uptake

Once steady state conditions had been achieved in continuous culture, samples of cells were removed from the chemostat and collected by centrifugation (4000 x g for 5 min). Cells were then washed twice with distilled water and resuspended in 50 mM MES buffer containing 50 mM glucose, pH 5.5 at 30 ^{o}C. The cell density was adjusted to 0.15 mg ml^{-1} for ^{54}Mn uptake assays and 0.5 mg ml^{-1} for Cu^{2+} and

non-radioactive Mn^{2+} uptake assays. Cells were then allowed to equilibrate in this medium for 10 min prior to the addition of metal ions. ^{54}Mn was added to give a final concentration of 10 nM, Cu^{2+} was added to 10 μM, and non-radioactive Mn^{2+} was added to 50 μM. Metal uptake assays are as previously described in Chapter 3.

10.3. Results

10.3.1. Continuous culture

Although it was experimentally difficult to assess the trace Mn^{2+} present in the low-Mn^{2+} medium due to medium impurities, an estimation of less than 0.05 μM Mn^{2+} was obtained by concentration of the medium using rotary evaporation and analysis by atomic absorption spectroscopy. Under steady state conditions, growth in basic medium (0.45 μM Mn^{2+}) and in low- (< 0.05 μM Mn^{2+}) and high-Mn^{2+} (100 μM Mn^{2+}) media was glucose-limiting, the cell yield being 4.4 mg ml^{-1} in each case. Figure 10.3.1. shows the effect of Mn^{2+} in the growth medium on ^{54}Mn uptake *via* the Mn^{2+} specific transport system. Cells grown in basic medium and low-Mn^{2+} accumulated sustantially greater amounts of Mn^{2+}, having initial uptake rates of 0.55 ± 0.11 nmol (g dry wt)$^{-1}$ min^{-1} and 0.69 ± 0.16 nmol (g dry wt)$^{-1}$ min^{-1} respectively, compared to an initial rate of only 0.05 ± 0.01 nmol (g dry wt)$^{-1}$ min^{-1} for cells grown in 100 μM Mn^{2+}.

Growth of cells in basic medium (0.54 μM Cu^{2+}) and high-Cu^{2+} medium (25 μM Cu^{2+}) was glucose-limited. However when transferred to Cu^{2+} extracted medium, glucose appeared in the effluent at a concentration of 0.45 mM and the cell yield was reduced from 4.4 mg ml^{-1} to 2.6 mg ml^{-1}. Cells grown in batch culture in basic medium amended

with 50 mM MES buffer exhibited no significant difference in mean generation time as compared to cells batch grown in high-Cu^{2+} medium. Therefore it is unlikely that Cu^{2+}, at the concentrations used in these experiments, had any significant effect on growth. Cells grown in Cu^{2+}-limiting, basic and high-Cu^{2+} media exhibited very similar uptake profiles (Fig. 10.3.2.). Cells grown in high-Cu^{2+} medium contained a higher basal Cu^{2+} content, however the initial Cu^{2+} uptake rates for cells grown in Cu^{2+}-limiting, basic and high-Cu^{2+} media were 0.71 $\pm$ 0.06 μmol (g dry wt)$^{-1}$ min^{-1}, 0.74 $\pm$ 0.08 μmol (g dry wt)$^{-1}$ min^{-1} and 0.71 $\pm$ 0.03 μmol (g dry wt)$^{-1}$ min^{-1} respectively. Thus, varying the Cu^{2+} levels of the growth medium appears to have no effect on the rate of Cu^{2+} uptake.

Figure 10.3.3. describes the effect of varying Mg^{2+} concentration on the cell yield of *C.utilis* following 24 h growth in batch culture. At medium Mg^{2+} concentrations below 75 μM, growth limitation occurs as indicated by a decrease in the cell yield. From this graph, a Mg^{2+} concentration of 25 μM was selected as a suitable concentration at which to perform Mg^{2+}-limiting continuous culture experiments. At this Mg^{2+} concentration, the cell yield was approximately 50 % of that attained by batch cultures containing greater than 100 μM Mg^{2+}.

Cells grown in basic medium (6.5 mM Mg^{2+}) were glucose-limited whilst cells cultured in low-Mg^{2+} medium (25 μM Mg^{2+}) were Mg^{2+}-limited with glucose present in the medium outflow and gave a cell yield of 2.1 mg ml^{-1}. On exposure to 50 μM Mn^{2+}, Mg^{2+}-limited cells rapidly hyperaccumulated Mn^{2+} far in excess of the level attained by cells grown in basic medium (Fig. 10.3.4.). At this high divalent cation concentration, uptake of Mn^{2+} would be *via* the non-specific divalent cation carrier. Thus, the effects observed in this instance reflect

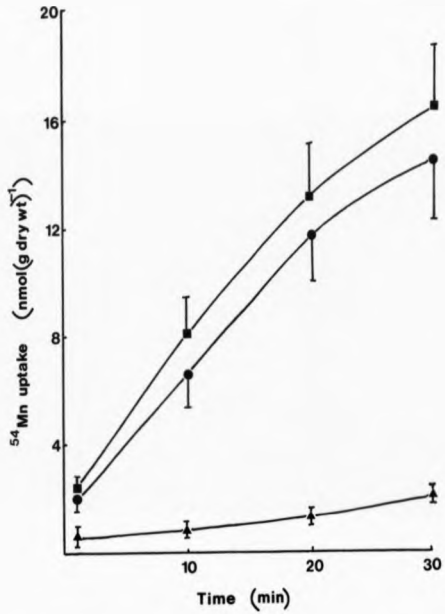


Figure 10.3.1. Repression of ^{54}Mn uptake. ^{54}Mn uptake by cells grown in (■) low-Mn^{2+} (< 0.05 μM); (●) basic medium (0.45 μM); (▲) high-Mn^{2+} (100 μM). Values shown are the mean ± SE of 3 experiments.

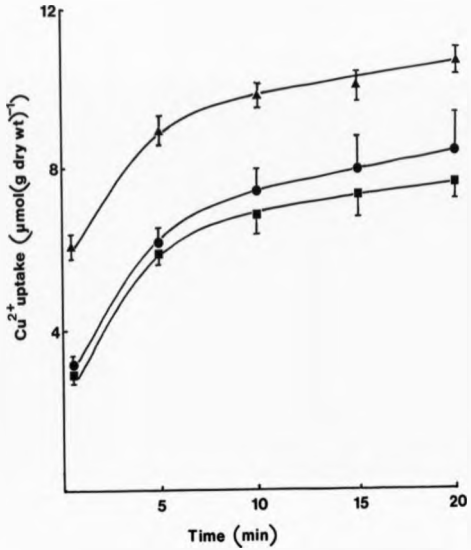


Figure 10.3.2. Constitutivity of the Cu^{2+} transport system. Cu^{2+} uptake by cells grown in (■) Cu^{2+}-limiting medium; (●) basic medium (0.54 µM); (▲) high-Cu^{2+} (25 µM). Values shown are the mean ± SE of 3 experiments.

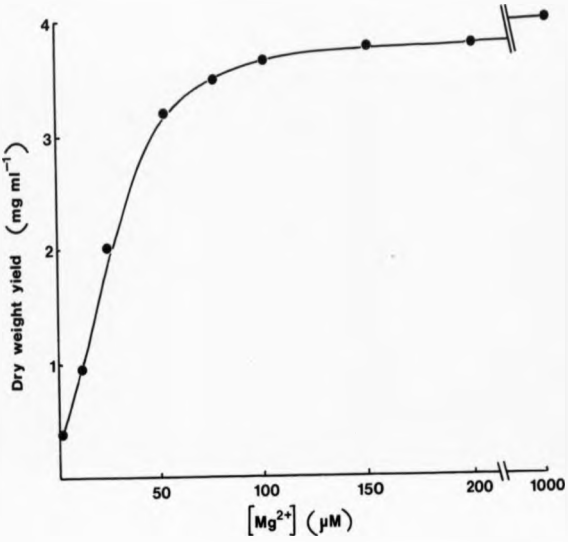


Figure 10.3.3. Effect of Mg^{2+} in the growth medium on cell yield following 24 h growth of *C.utilis*.

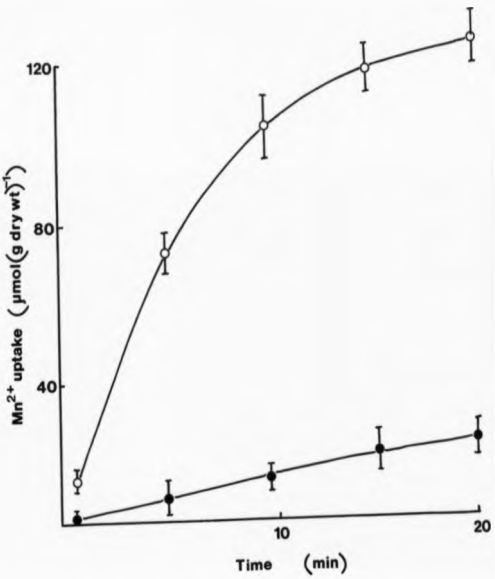


Figure 10.3.4. Mn^{2+} uptake from 50 μM Mn^{2+} by cells grown in basic medium (6.5 mM Mg^{2+})(●), in Mg^{2+}-limiting medium (25 μM Mg^{2+})(○). Values shown represent the mean ± SE of 3 determinations.

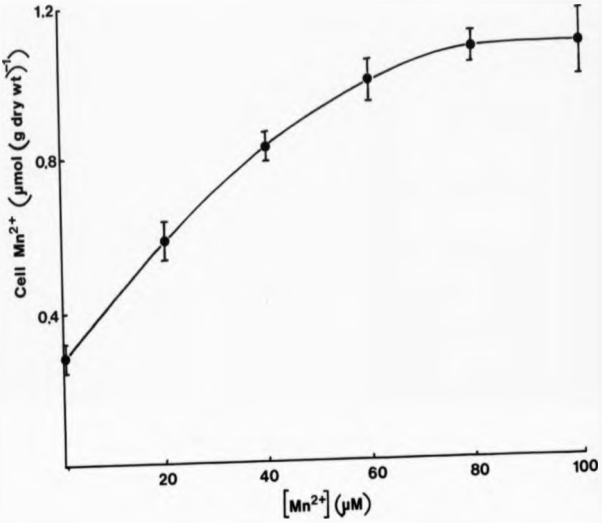


Figure 10.3.5. Effect of Mn^{2+} in the growth media on the intracellular Mn^{2+} content. Values shown represent the mean ± SE of 3 experiments.

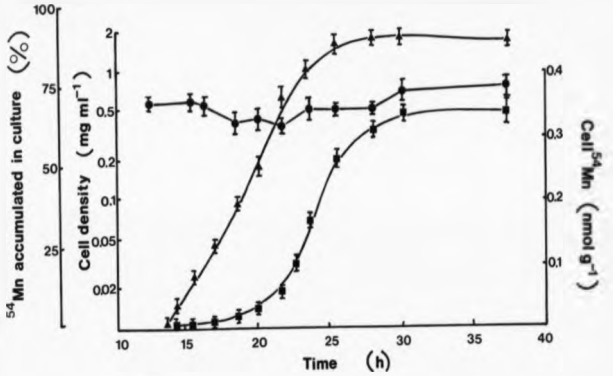


Figure 10.3.6. ^{54}Mn accumulation during growth in batch culture, with pH control. (▲), cell density of culture; (■), total ^{54}Mn accumulated by culture; (●), cellular ^{54}Mn content. Values shown are the mean ± SE of 3 experiments.

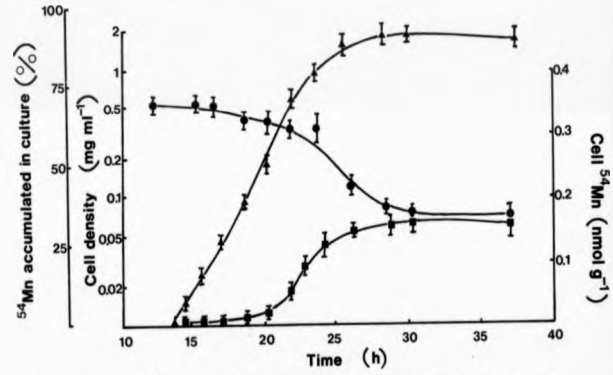


Figure 10.3.7. ^{54}Mn accumulation during growth in batch culture, no pH control. (▲), cell density of culture; (■), total ^{54}Mn accumulated by culture; (●), cellular ^{54}Mn content. Values shown are the mean ± SE of 3 experiments.

changes in the capacity of this transport system.

10.3.2. Batch culture

Cells were batch grown in media containing various Mn^{2+} concentrations up to 100 μM and harvested following 24 h growth. No effect on cell yield was observed. Figure 10.3.5. describes the relationship between external Mn^{2+} in the growth media and the cell Mn^{2+} content. On increasing the external Mn^{2+} concentration, the cell Mn^{2+} also increases until a value of around 1.1 μmol (g dry wt) is attained.

^{54}Mn accumulation during growth in batch culture is shown in Figure 10.3.6. The cell ^{54}Mn content remained fairly constant throughout the the growth period, despite the decreasing availability of ^{54}Mn in the growth media. It should be noted that the medium was closely maintained at pH 6.0 during this time by the addition of KOH. Failure to do so resulted in a drop in pH to 3.8 over 3.5 h during late-exponential phase coupled with a 50 % reduction of the cell Mn^{2+} (Fig. 10.3.7.). This dilution effect may well be due to reduced Mn^{2+} uptake under low pH conditions during culture growth, emphasising the importance of adequate pH control during batch culture studies.

10.4. Discussion

There appears to be a considerable difference between the mode of regulation of the Mn^{2+}-specific transporter and the non-specific Mg^{2+} carrier and that of the Cu^{2+} transport system. Uptake of ^{54}Mn *via* the Mn^{2+}-specific system was repressed in cells grown in high-Mn^{2+}. Uptake from high concentrations of Mn^{2+} by way of the Mg^{2+} transport

system was also regulated with respect to the Mg^{2+} concentration of the growth medium. In the latter case, the use of Mn^{2+} is as a probe for the non-specific Mg^{2+} carrier. The observed reduction in the rate of Mn^{2+} transport is analogous to a 3 to 6-fold reduction in the maximal Mn^{2+} uptake rate reported in high-Mn^{2+}-grown *Bacillus subtilis* cells (Eisenstadt *et al*, 1973) where it is suggested this effect may be due to fewer or altered Mn^{2+} transport carriers being synthesised during growth in high-Mn^{2+}.

There are a variety of strategies by which an organism might increase transport of a growth limiting nutrient such that a maximum yield factor is obtained under the prevailing conditions (Harder & Dijkhuizen, 1983). The rate of transport could be increased by synthesis of more of the existing uptake system which would increase the V_{max} and enable an increase in transport at low (subsaturating) solute concentrations. Alternatively, an organism may possess the capacity to synthesise a different high-affinity uptake system to scavenge the available nutrient. A further possibility is that a change in the kinetic properties of the uptake system may occur, affecting parameters such as the binding affinity of the solute for the transport site, the stoichiometry of the transport mechanism or the carrier activity (Harder & Dijkhuizen, 1983).

Silver and Jasper (1977) proposed a model for the regulation of Mn^{2+} transport in bacteria whereby Mn^{2+} starvation induces the synthesis of additional Mn^{2+} carriers in the cell membrane. This is manifested in an increase in V_{max} in the absence of a change in K_t. However, conditions of high exogenous Mn^{2+} would favour the synthesis of an intracellular protein inhibitor which could then inactivate the Mn^{2+} transport system. This protein inhibitor would be specific for the

Mn^{2+} transporter and might exert its action by either binding to part of the uptake system, thereby blocking its transport function, or if the inhibitor is a specific protease, by breaking down part of the carrier protein. In view of the similarity with bacterial Mn^{2+} transport, it is quite possible that such a mechanism of control may operate to regulate Mn^{2+}-specific uptake in *C.utilis*. To the best of the author's knowledge, there is no data on yeasts or fungal cells currently available with which to compare the regulation of the non-specific Mg^{2+} transporter in cells grown in high or low Mg^{2+}. It would not be unreasonable to assume that some mechanism, similar to that described above, may increase the capacity of cells to accumulate Mg^{2+} under conditions when the availability of this important divalent cation becomes growth-limiting. Hence, although the primary aim of this response to limitation is to increase Mg^{2+} uptake, a secondary effect would be the increase in transport of alternative substrates, such as Mn^{2+}.

There are two further possible explanations of the apparent increase in transport observed in Mg^{2+}-limited cells which, whilst their likelihood does not seem as great, are also worthy of consideration. Changes in the surface structure of the cell may arise during nutrient limitation which facilitate an improvement in the binding of the nutrient. This has been observed with Mg^{2+}-limited cultures of *B.subtilis* in which alterations in the cell wall teichoic acid content and composition led to a substantially greater capacity of the walls to bind Mg^{2+} (Tempest & Neijssel, 1976). This seems unlikely in the present study as the uptake profiles are not indicative of instantaneous surface binding of metal. In addition, growth limitation by Mg^{2+} (as with K^{+}-limitation) generally gives rise to an increase in the cellular respiration rate (Harder &

Dijkhuizen, 1983). This is because the cells must expend more energy to maintain the transmembrane Mg^{2+} gradient which will be maximised under conditions of Mg^{2+}-limitation. The increase in transport seen in Mg^{2+}-limited *C.utilis* may result directly from this stimulation of respiratory activity. However, this would not explain why, under glucose-limited conditions, regulation of Mn^{2+} uptake occurred.

Although it is not possible to directly compare uptake by Cu^{2+}-limited cells with cells grown in low Mn^{2+} as Mn^{2+}-limitation was not achieved, by comparing uptake by cells grown in high-Mn^{2+} and high-Cu^{2+} and in basic medium, major differences can be seen. In contrast to the observed hyperaccumulation of Zn^{2+} by *C.utilis* cells grown in Zn^{2+}-limiting medium (Lawford *et al*, 1980), there was no detectable difference in the Cu^{2+} uptake rate in Cu^{2+}-limited cells. Similarly, in high-Cu^{2+} medium, no apparent repression of Cu^{2+} transport occurred. It is possible that the reduced cytochrome aa_3 content resulting from Cu^{2+}-limitation in yeasts (Light, 1972) may directly or indirectly impair Cu^{2+} uptake thus masking any elevated transport, but in view of the almost identical initial uptake rates for all three Cu^{2+} conditions this seems most unlikely. These results indicate that the Cu^{2+} transporter in *C.utilis* is a constitutive system. Silver and Clark (1971) described a constitutive Mg^{2+} transport system in *E.coli*, however cell Mg^{2+} requirements are generally much greater than the requirement for Cu^{2+} and a quite separate and repressible Mg^{2+} transporter has also been demonstrated in *E.coli* (Nelson & Kennedy, 1972).

It is possible that one implication of this lack of regulation of Cu^{2+} uptake is the relatively high sensitivity of *C.utilis* to Cu^{2+} poisoning. When cells are subjected to high external Cu^{2+}

concentrations, subsequent uncontrolled internalisation of Cu^{2+} would rapidly kill the cells.

The limited increase, prior to levelling-off, of the cell Mn^{2+} content during batch growth in widely varying Mn^{2+} concentrations provides an indication of the ability of *C.utilis* to regulate its intracellular Mn^{2+}. It seems quite likely that the observed repression of Mn^{2+} transport during growth in high-Mn^{2+} may play a vital role in Mn^{2+} homeostasis in this yeast and the importance of such an effect can be seen here. Flatman (1984) suggested the existence of powerful Mg^{2+} regulatory systems in the membrane of *E.coli*, as the cell Mg^{2+} content only doubles when the medium Mg^{2+} concentration is raised several thousand-fold. There is little information describing the control of cytosolic metal levels by transport modulation in yeasts or fungi. However, the maintenance of low intracellular metal concentrations in the presence of high exogenous metal is well documented. Cell Cu^{2+} and Zn^{2+} in *Penicillium ochro-chloron* remained virtually constant in high external metal concentrations and the intracellular Mg^{2+} content of *S.cerevisiae* and Co^{2+} content of *Neurospora crassa* both levelled-off when the external divalent cation concentration rose to millimolar levels (Conway & Beary, 1958, Venkateswerlu & Sastry, 1970).

Fluctuations in cellular metal levels during batch growth have been observed for Zn^{2+} in *C.utilis* (Failla & Weinberg, 1977). However, Mn^{2+} uptake in the same species in this study does not appear to be under such regulation during batch culture, the cell ^{54}Mn level remaining constant around 0.35 nmol (g dry wt)$^{-1}$. Attempts to culture cells in the manner described by Failla and Weinberg (1977) resulted in a pH drop to 3.8 during late-exponential phase of growth

necessitating close pH control during this period. Under these conditions, Mn^{2+} uptake would be severely limited and fluctuations in the cell Mn^{2+} content, similar to those described by others, became evident. However this effect may be considered as being a direct result of the decrease in pH and not due to some regulatory mechanism within the cells.

Cu^{2+} accumulation in *A.niger* was rapid in lag phase, whilst during the subsequent linear growth phase the mycelial Cu^{2+} content dramatically reduced on a per gram dry weight basis (Townsley & Ross, 1986). This fluctuation was not due to the decrease in pH but was attributed to Cu^{2+}-complexation as a result of organic acid synthesis and secretion by the fungus. However it is noted that energy-dependent transport of metals in this fungus could not be demonstrated and would, in all probability be masked by the extensive binding of metal ions to the fungal cell walls. Thus, it is difficult to postulate whether the decrease in mycelial Cu^{2+} content following lag phase is a true reflection of the intracellular Cu^{2+} content or simply reflects the wall-bound Cu^{2+}. Evidence for the latter is provided by Walker (1985) who describes the greatly elevated capacity of the cell wall of *Schizosaccharomyces pombe* to bind Mg^{2+} during lag phase.

The study of ^{54}Mn uptake during batch culture clearly shows the physiological advantage of possessing such a Mn^{2+} scavenging system when considering the remarkable ability of *C.utilis* to accumulate almost 70% of the available ^{54}Mn from an initial concentration of 1nM in the presence of $4x10^6$-fold Mg^{2+}, $1.1x10^6$-fold Ca^{2+}, $6x10^3$-fold Zn^{2+} and 400-fold Cu^{2+}.

Thus in conclusion, Cu^{2+} transport in *C.utilis* does not appear to be regulated with respect to external Cu^{2+} concentrations and behaves in a different way to Mn^{2+} and Zn^{2+} transport and divalent cation uptake *via* the Mg^{2+} carrier. In addition to the observed repression of Mn^{2+} uptake under high-Mn^{2+} conditions, it seems likely that cells would hyperaccumulate Mn^{2+} in a similar manner to Zn^{2+} accumulation in Zn^{2+}-limited cells if Mn^{2+}-limiting conditions could be achieved. Furthermore, this influx regulation may well be involved in the maintenance of intracellular Mn^{2+} concentrations, which appear to remain constant throughout the growth curve during batch culture. This regulatory ability is of importance in many natural ecosystems where the capacity to compete effectively for the limited available nutrients may often mean the difference between survival and death of an organism. In the presence of excess Mn^{2+} in the growth media, *C.utilis* appears to be equally effective in preventing the build-up of high intracellular metal levels demonstrating the capacity of yeasts for divalent cation homeostasis.

11. GENERAL DISCUSSION

The purpose of this chapter is to draw-together the detailed discussions and conclusions contained within the experimental sections, and to identify future avenues of research which have been prompted by this study.

The study of Cu^{2+} and Mn^{2+} toxicity to *Candida utilis* was a useful and important prerequisite to transport studies of these metals. This is one aspect which has been largely ignored in a number of studies of yeast Cu^{2+} uptake. (Khovrychev, 1973, Wakatsuki *et al*, 1979). Whereas other authors have simply monitored cell viability as a measure of toxicity, it is apparent that sub-lethal concentrations of metals may firstly inhibit respiratory activities and therefore most probably, transport processes. Hence this preliminary data gave some idea of acceptable working levels of metals at which to perform uptake experiments. Cu^{2+} was considerably more toxic to *C.utilis* than Mn^{2+}, which exhibited little toxicity. The exact nature of the sites of action of Cu^{2+} poisoning in *C.utilis* could not be ascertained from this study although it seems likely that Cu^{2+} may firstly be transported inside the cell where it then binds to anionic sites on protein moieties. At higher Cu^{2+} concentrations, membrane permeability was affected as indicated by loss of cellular K^{+}.

Uptake of Mn^{2+} and Cu^{2+} in *C.utilis*, in accordance with a number of previous reports (Borst-Pauwels, 1981) was a biphasic process. The initial phase involved a rapid and reversible binding of metal ions to anionic groups at the surface of the cell, presumably onto the cell wall and outer membrane surface. This binding appeared to be non-specific and was independent of the involvement of metabolism.

This was followed by a slower and more progressive uptake of the cations into the interior of the cell. This intracellular transport was energy-dependent and was specific in as much as the transport systems present exhibit different specificities for different divalent cations.

The role of metal-wall binding was not clear and may have been fortuitous. It is possible that it may serve to preconcentrate trace metals from the environment such that the available metal concentration in the vicinity of the uptake system is effectively raised, allowing for greater transport. Using such analytical techniques as electron spin resonance (ESR) and nuclear magnetic resonance, information on metal coordination to specific functional groups has now been obtained. Whilst there is no data to account for the Cu^{2+}-binding observed here, Cu^{2+}-binding sites on cells of *Rhizopus arrhizus*, *Epicoccum* sp., *Penicillium* sp. and the alga, *Chlorella regularis*, have been identified and amine-nitrogen groups implicated. These reports are reviewed by Townsley (1985). The metal-binding groups of the cell wall of *C.utilis* could thus be determined by mechanical disruption of cells, selective enrichment and extraction of the cell wall fraction, followed by addition of metals and ESR analysis. Work of this nature may well explain the much greater affinity of Cu^{2+} for wall sites than Mn^{2+} noted in this study. It is possible that additional, different binding sites are involved in Cu^{2+} coordination.

The plasma membrane of *C.utilis* appeared virtually impermeable to Mn^{2+} and Cu^{2+} and no evidence of diffusion was observed either from the external medium into cells or from metal-laden cells back to the extracellular milieu. This finding is not surprising in view of the

vast majority of reports of metal ion uptake demonstrating energy dependence, although several authors have indicated a passive transport role in fungi (Broda, 1972, Puckett *et al*, 1973, Shatzman & Kosman, 1978).

Natural environments will very rarely contain those divalent cations which are essential for growth at a potentially maximum rate (Tempest & Neijssel, 1978) and thus microbes have evolved the ability to concentrate these essential ions to intracellular levels greatly in excess of those present in the environment. The concentration ratios for Mn^{2+} and Cu^{2+} uptake in *C.utilis* (180:1, inside:outside and 90:1, inside:outside respectively) determined for the particular uptake conditions imposed, clearly demonstrate the usefulness of such metabolism-dependent processes. However, there are several considerations to be borne in mind. The exact nature of the effective concentration gradient across the cell membrane is not clear.

Cytoplasmic sequestration of the metal would greatly decrease the freely available internal metal concentration. Similarly, localised concentration of metal at the cells external surfaces would also decrease the concentration gradient. One possible method to circumvent this problem would be the use of ion-selective microelectrodes inserted through the cell membrane. The technology to undertake work of this nature is now available and practical details of cation-sensitive microelectrodes are described by Clarkson (1974) and Kotyk and Janacek (1975). Successful insertion of microelectrodes has been reported in *Neurospora crassa* (Sanders *et al*, 1983, cited by Eddy & Hopkins, 1985). However, this ability to accumulate nutrients against a concentration gradient provides a

vital demarcation between facilitated diffusion and active transport. Active transport implies uptake against a gradient whilst facilitated diffusion describes carrier-mediated translocation down a concentration gradient. It will be noted that avoidance of the term "active" has been made throughout the text and the distinction by Scarborough (1985) of "transport coupled to electrochemical ion gradients or chemical transformations", adopted. Rosen and Kashket (1978) have suggested that distinctions based on transport up or down a concentration gradient may no longer be necessary and have described the case of sugar transport in *Streptococcus lactis* and *Escherichia coli*. Energy dependent transport of sugars can occur either up or down the sugar concentration gradient, depending entirely on the asymmetry imposed by a proton gradient which provides the driving force for the sugar/H^+ symport. Movement of sugars down the concentration gradient could not occur unless an energy source was added which then supplied the proton gradient. However in *C.utilis*, whilst there remain reservations about the validity of the observed concentration gradients, in view of the extremely low medium metal concentrations from which the cell must scavenge its requirements, and of the relatively high basal cell content of these metals, it seems likely that energy-dependent uptake of Mn^{2+} and Cu^{2+} is against a concentration gradient.

The inhibition of metal uptake in the presence of specific and non-specific inhibitors of metabolism demonstrated beyond doubt the involvement of energy coupling to metal transport processes. The reduced sensitivity of Cu^{2+} uptake to CCCP was of interest and gave the first indications that the Cu^{2+} uptake mechanism differed from that of Mn^{2+} uptake. This raised the possibility that either the energy input to Cu^{2+} transport or the Cu^{2+} transport process itself

was implicitly different. There is great scope for investigations of this type into the coupling mechanisms linking the energy source and transporter. Little information is presently available on the molecular basis of microbial metal ion uptake systems and their driving forces, unlike other widely investigated carriers, such as amino-acid or sugar translocators. Recently acquired knowledge of the energy-requirements of metal transport systems, together with our clearer understanding of yeast and bacterial metabolism should now facilitate work of this nature.

Prior to this study, the picture of divalent cation uptake in *C.utilis* was not clear. It appeared that, as in most yeast species studied so far, a general, non-specific divalent cation transport system existed which was capable of translocating most divalent cations, some with greater affinities for the system than others (Fuhrmann & Rothstein, 1968, Borst-Pauwels, 1981). The affinity for Mg^{2+} was particularly high and these observations are closely paralleled in bacterial studies (Silver, 1978). Whilst these studies were all carried out using high (millimolar) concentrations of divalent cations, it was generally assumed that in a more physiological situation, transport of all these essential cations would be *via* this route. What was not clear was how cells might transport trace cations, such as Mn^{2+}, Zn^{2+} and Cu^{2+}, as lower affinity substrates, when in the presence of concentrations of a higher affinity substrate, Mg^{2+}, many thousand-fold greater. This situation would arise in most growth media. The presence in bacteria of a series of highly specific metal uptake systems, coupled with the data on Zn^{2+}-specific uptake in *C.utilis* (Failla *et al*, 1976) indicate that specific systems of this nature may exist in yeasts. This is in accordance with Silver's proposal (Silver, 1978) that each

essential metal will have specific system to facilitate its transport. As these systems are generally undetected at high metal concentrations, it is proposed that most previous yeast metal uptake studies described the Mg^{2+} uptake system. Thus, it was of great interest to observe that Mn^{2+} and Cu^{2+} upake from very low concentrations of these metals appeared to be by way of highly specific processes. It is postulated that these specific systems would operate under physiological conditions to scavenge the trace amounts of Mn^{2+} and Cu^{2+} present in the external milieu that the cell requires. The Zn^{2+} specific system already observed by Failla *et al* (1976) would present a further example of this in *C.utilis*.

The findings of the present study have been combined with the data of Failla *et al* (1976) to produce a picture of our present knowledge of divalent cation transport in *C.utilis*. This is shown in Figure 11.1. At trace concentrations, Zn^{2+}, Mn^{2+} and Cu^{2+}, enter the cell through three separate transport processes, specific to each cation. At higher concentrations, when the systems have reached saturation with their respective solutes, these metals are capable of entering as low affinity substrates of the Mg^{2+} uptake system. In most environmental situations this would probably not occur. However when cells are exposed to high concentrations of metal pollutants resulting from anthropogenic activities, the role of the Mg^{2+} uptake system in accumulating high cellular levels of divalent cations would probably be a profound factor in metal toxicity. The specific metal transporters may also have an important role in the relationship between uptake and toxicity. One possible explanation for the high toxicity of low levels of Cd^{2+} may be the fortuitous concentration of Cd^{2+} within cells by the Zn^{2+} uptake system, due to the chemical and physical similarities between these two cations (Failla *et al*, 1976).

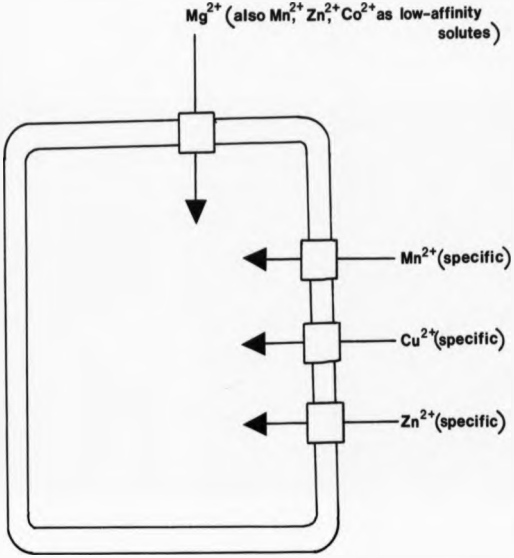


Figure 11.1. A current view of the divalent cation transport systems of *C.utilis*.

The high specificities exhibited by such systems may be significant in the concentration in microbial cells of ^{54}Mn from radioactive fallout. The clear ability of microbes to selectively accumulate ^{54}Mn would result in the potentially harmful incorporation of this nuclide within food chains (Silver & Jasper, 1977).

The presence of parallel systems for metal uptake in bacteria as outlined in chapter 6, may be an indication of the many free-living cell types which possess specific transport mechanisms for essential nutrient cations. Indeed, one might use this criteria to ascertain the essentiality of a metal ion. According to Silver *et al* (1970), it proved impossible to create conditions such that a Mn^{2+} requirement might be found in *E.coli*, however the existence of a specific Mn^{2+} transport system was suggestive of a vital role for Mn^{2+}.

The high degree of specificity exhibited by the low level Mn^{2+} uptake mechanism is certainly suggestive of a complex molecule, quite possibly a protein, being involved in the metal binding site at the membrane outer surface. It would be an elegant procedure if this compound could be isolated in a similar manner to the isolation and identification of sugar permeases. There may be several fruitful lines of investigation. The use of mutants which are defective in a particular transport system provides a powerful experimental tool with which to study uptake. Nelson and Kennedy (1972) and Park *et al* (1976) successfully used transport-mutants to identify two Mg^{2+} uptake systems in *E. coli*, one system was repressible and highly specific for Mg^{2+}, whilst the second was non-specific and constitutive. It may be possible, by sub-lethal u.v. irradiation of *C.utilis*, to produce a mutant defective in the specific Mn^{2+}

transporter. By gel electrophoresis of the membrane proteins of this strain, any protein differences to the wild-type could be detected. The presence of a transport protein might be shown by overlaying the gel-bound proteins with ^{54}Mn, washing and assaying fractions for radioactivity. Alternatively, when studying a repressible system, quantitative determinations of membrane proteins from repressed and depressed cells might reveal the presence of a protein implicated in uptake. Whilst these proposals may be confounded by practical difficulties, future investigations of this little-studied area would appear to have great potential.

A detailed examination of the kinetic parameters of the various uptake processes, as described in Chapter 7 confirmed the presence of three distinct transporters in *C.utilis*. The non-comparability of kinetic data from different laboratories has proved a major obstacle in identifying metal transport systems and this is evident in a review of kinetic ion uptake data in yeasts (Borst-Pauwels, 1981). Indeed, it would appear to be of great value to propose a standardised incubation medium with defined cellular pretreatment conditions for use in metal uptake experiments. However, all kinetic parameters obtained in this study were directly comparable, the uptake conditions being identical. The difference in transport constants of the non-specific divalent cation transporter and the Mn^{2+}-specific system was many orders of magnitude. Similarly, the affinities of other divalent cations for the Mn^{2+}-specific carrier were many times lower than the affinity for Mn^{2+}ions. Thus the vast difference in Mn^{2+} uptake *via* the two systems is evident. The kinetic data also gave some indication of the relative affinities of Mn^{2+}, Co^{2+} and Zn^{2+} for the non-specific Mg^{2+} uptake system and these values compared favourably with the available literature (Silver,

1978, Borst-Pauwels, 1981).

The specific uptake of Cu^{2+} was of particular interest as, except for several reports from Gadd's laboratory using metal tolerant fungal species (Gadd & Griffiths, 1980, Gadd *et al*, 1984a, Gadd & White, 1985, Gadd & Mowll, 1985), there is little reliable data on the uptake of non-toxic concentrations of Cu^{2+} in yeasts and fungi. It is even less clear whether Cu^{2+} enters cells *via* the non-specific Mg^{2+} uptake system or by way of some other system as virtually no competition studies have been performed. Thus, as with Mn^{2+}-specific transport, the specific Cu^{2+} uptake observed in *C.utilis* was a somewhat novel occurrence. In view of the plethora of reports of metal uptake in *S.cerevisiae via* the non-specific Mg^{2+} transporter, there is now a case for performing low-level metal uptake experiments to determine whether or not specific uptake systems for Mn^{2+}, Cu^{2+} and Zn^{2+} also exist in this yeast species. As *S.cerevisiae* is the most widely studied and well-known of yeasts, this information would provide a useful contribution to our knowledge of yeast physiology.

The efflux of metals from cells is still not a clearly defined phenomenon and it remains unknown if the putative Cu^{2+} efflux system proposed by other authors (Silver, 1978, Eilam, 1982) is responsible for Mn^{2+} efflux in *S.cerevisiae* (Theuvenet *et al*, 1986). Efflux analysis is greatly complicated by the ability of microbes to reaccumulate metal generally at a faster rate and complex flux studies would be required to obtain further data. Again, the use of transport-impaired mutants might provide one answer to this as might the addition of metal chelators to prevent subsequent metal reaccumulation. The stoichiometric efflux of protons concomitant with Mn^{2+} uptake *via* the Mg^{2+} transport system was an unusual

observation in that previous authors have commonly detected a release of cellular K^+ in response to divalent cation uptake. However, these observations were for *S.cerevisiae* and it was not always clear whether this was due to reduced membrane permeability resulting from metal toxicity. Ejection of protons, presumably by way of a plasma membrane ATPase system (Goffeau & Slayman, 1981), coupled to metal influx may well provide some indication of the involvement of protons in the driving force for metal uptake. Indeed, there appears to be an intimate relationship between divalent cation influx and movement of monovalent cations which is worthy of further study.

The effect of factors such as pH, K^+ and phosphate in the external medium all served as useful criteria with which to further characterise each system studied. Not surprisingly, varying the pH exerted a similar effect on all three uptake systems examined and this response is well-documented in yeasts (Borst-Pauwels, 1981). Phosphate was shown to dramatically stimulate Mn^{2+} uptake *via* the Mg^{2+} uptake system and this is in agreement with the findings of Rothstein *et al* (1958). The lack of similar stimulation of Cu^{2+} uptake by phosphate provides further evidence of the multiplicity of transport systems. Whilst results of this type do not, in themselves, constitute conclusive evidence that separate systems do exist, they do provide useful supplementary information to competition studies. On the basis of the collated data as a whole, the evidence to support the postulation that essential metal influx in *C.utilis* is by way of specific uptake systems remains convincing.

Silver (1978) has pointed out the growth advantages that substrate specific transport systems provide under these conditions where the availability of essential micro-nutrients limit growth. Such systems

generally have very low K_t values and very high specificity to exclude related macro-nutrients, such as Mg^{2+}. It is further stated that these micronutrient uptake systems are generally subject to repression and induction or a feedback inhibition - type regulatory control. This modulation serves to protect cells from accumulation of high toxic levels of metal during rare conditions when their abundance is high.

Mn^{2+}-specific uptake in *C.utilis* is strongly inhibited following growth in high Mn^{2+} conditions representing repression of normal trace scavenging transport systems. The anomalous constitutivity of the Cu^{2+}-specific transporter is hard to explain and merits further attention, however little information is currently available regarding regulation of metal transport systems. Transport of divalent cations in growing cells, as opposed to non-growing, metabolising suspension of cells, is a further area where few physiological studies have been undertaken. The data for Mn^{2+}-specific uptake in *C.utilis* is suggestive of a constant level of uptake throughout the growth curve, although Townsley & Ross (1985) have cautioned the assumption that uptake in both types of cells is identical.

In the introduction the basic premise was put forward, as hypothesised for bacterial transport (Silver & Jasper 1977) that a series of high affinity, highly specific metal cation uptake systems will exist for the purpose of translocating essential micro-nutrients. It appears, on the basis of this study, that the postulate holds true for Mn^{2+} and Cu^{2+} uptake in *C.utilis*. In addition, as shown in other yeast species, a general, non-specific Mg^{2+} uptake system is present which is also capable of translocating

other divalent cations. Although very considerable differences in cell biology and physiology exist between prokaryotic and eukaryotic cells, nevertheless, the study serves to demonstrate that lessons can be learned from extrapolating data from prokaryotic systems to eukaryotic systems.

To the author's knowledge, this is the first report of specific Mn^{2+} and Cu^{2+} uptake systems and their regulation in a yeast species and this data together with the findings of Failla *et al* (1976) on Zn^{2+}-specific transport in *C.utilis*, provide an important addition to our knowledge of the accumulation of essential trace metals. To conclude, it seems likely that systems of this nature may be found in most microorganisms and hence there is great scope for studies of this kind in other yeast species and fungi. Furthermore, by extending our knowledge of macro- and micro-nutrient transport systems we will obtain an insight into the ways in which yeasts acquire both essential and non-essential, toxic elements under natural physiological conditions.

APPENDIX I

AN ESTIMATION OF THE SURFACE POTENTIAL OF *C.UTILIS* CELLS USING 9-AMINOACRIDINE AS A PROBE

A.1. Introduction

A phenomenon of negatively charged biological membranes is the presence of a double layer of ionic charges associated with them (McLaughlin *et al*, 1971). This results from the electrostatic attraction of cations to negative charges on the membrane (thus increasing their effective concentration at the membrane) and also from the thermodynamic tendency of these cations to diffuse away from the surface. Hence at equilibrium, a stable diffuse double layer exists. The differential effects of monovalent and divalent cations in this double layer on the surface potential of the membrane has been quantitatively described (Barber *et al*, 1977) and the derived equation can be used to determine the relationship between salt concentration and the magnitude of the surface potential.

There have been several methods used to estimate the negative potential at the surface of yeast cells. One report used the zeta-potential of cells as a measure of the surface potential, as determined by particle electrophoresis (Borst-Pauwels & Theuvenet, 1984). However, the zeta potential is the potential difference at the surface of shear relative to the bulk medium and assumes that the plane of shear is at the membrane surface. With the rigid cell wall structure present at the yeast cell surface, this method will give an inaccurate picture of the potential at the membrane surface. Chow and Barber (1980) showed that a negatively-charged membrane surface

could quench fluorescence of 9-aminoacridine. This quenching effect was due to the electrostatic attraction of this monovalent cationic dye into the diffuse double layer adjacent to the negatively-charged surface. Addition of other cations could be used to relieve the quenching by decreasing the surface potential and displacing the dye from the diffuse layer. The fluorescence quenching could be used to quantify the surface charge density (and hence the surface potential) of the membrane by invoking the equation relating to the aforementioned differential effect of mono- and divalent cations on surface potential.

This latter method has been applied to several studies of *S.cerevisiae* (Theuvenet & Borst-Pauwels, 1980, Theuvenet *et al*, 1984). It was shown that the electrostatic potentials probed by the dye were directly related to the zeta potentials, yet were much higher and were of the same order of magnitude as the theoretically calculated discrete charge potentials experienced by cation transporters in the membrane. Thus it was concluded that 9-aminoacridine could be used in whole cells as a convenient probe of the surface potential that affects ion transport kinetics. The ability of 9-aminoacridine to quantitatively determine the yeast surface potential could not be fully shown, as in addition to the simple electrostatic attraction between the dye and the membrane surface, other interactions were involved as was some dye binding to negative charges in the yeast cell walls.

This study was undertaken in order to estimate the magnitude of the surface potential of cells of *C.utilis* using 9-aminoacridine. This would then enable a closer appraisal of the possible effect that the surface potential may have on transport kinetics given the monovalent

and divalent cation concentrations used in Chapter 7.

A.2. Materials and methods

Fluorescence assays were carried out in a buffer containing 1 mM HEPES at a pH of 7.5 in a 2 ml assay volume. The cells used had been previously starved for approximately 24 h to minimise metabolic activity and were used at a final cell density of 2 mg dry wt ml^{-1}. 9-aminoacridine was added at a final concentration of 25 μM. Fluorescence of 9-aminoacridine was excited at < 390 nm using a Corning 7-39 filter and was measured perpendicular to the excitation beam at 450 - 500 nm with Wratten 45 and Corning 4-96 filters. The signal was amplified using a photomultiplier with Brandenburg power unit and recorded on a chart recorder. Addition of salts was made with microsyringes. The fluorescence emitted at each salt addition was expressed as a function of the maximal fluorescence achieved on adding 20 mM $MgCl_2$.

A.3. Results

As shown in Figure A.3.1, the progressive addition of the salts, $MgCl_2$ and KCl brings about an increase in 9-aminoacridine fluorescence. The efficacy of this quenching release is related to the valency of the cation, the divalent cation being more effective than the monovalent cation.

Chow and Barber (1980) have shown that from a derivation of the Gouy-Chapman theory relating surface charge density (q) to the

surface potential (Ψ), an expression could be used to determine q directly from the bulk concentrations of added cations:

$$q = - [2RTE_rE_0 (C^{+2} - 4C^{+}C^{++}) / C^{++}]^{1/2}$$

where R, the gas constant, = 8.314, T = 298 ^{0}K, E_r = 78.5 $\cdot$ E_0 (where E_0 is the permittivity of a vacuum) and C^{+} and C^{++} are the bulk concentrations of monovalent and divalent salts respectively.

Using this equation, which assumes that the surface charge density is the same when C^{+} gives an equal release of quenching of 9-aminoacridine as the equivalent C^{++}, the data in Figure A.3.1. can be substituted to calculate q for different levels of fluorescence quenching and the results of a typical experiment are shown in Table A.3.1. It can be seen that the charge densities obtained are around - 11 to - 15 μC cm^{-2}, however above a certain ionic threshold the value rises with the cation concentration.

Having obtained a value for the surface charge density, an estimation of the surface potential of the cell can be made using the expression described by Barber *et al* (1977) relating the two values at 25 ^{0}C:

$$q = 11.74 (C_a)^{1/2} \sinh (Z\Psi / 51.7)$$

where C_a = concentration in the bulk solution and Z = the charge on the cation. There are several assumptions made when using this expression which have been described in the above paper and are not dealt with here as, for practical purposes, it can be assumed that this is the best easily-available model to describe the above relationship. For example, assuming a charge density of - 12 μC

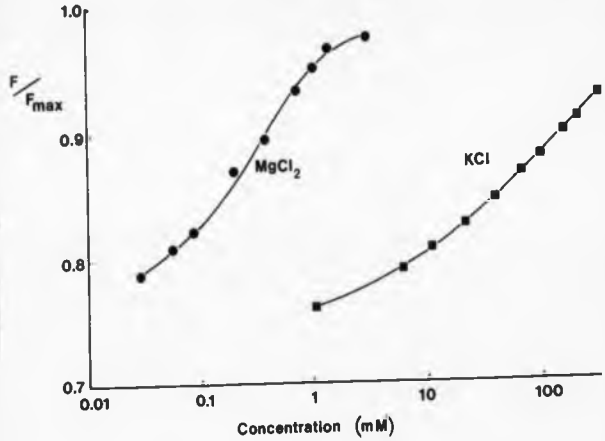


Figure A.3.1. Dependence of 9-aminoacridine fluorescence on the presence of mono- and divalent cations in *C.utilis* cells. The concentrations refer to the nominal concentrations of salts added.

Table A.3.1. Estimated charge densities for *C.utilis* cells

F / F_{max}	$MgCl_2$ (mM)	KCl (mM)	($\mu C/cm^2$)
0.72	0.03	12	-12.8
0.74	0.05	17	-14.0
0.82	0.10	19	-11.0
0.84	0.14	32	-15.7
0.86	0.20	55	-22.7
0.88	0.29	84	-28.7

cm^{-2}, at a monovalent cation concentration of 10 mM or a divalent cation concentration of 23.7 μM, the surface potential of the cell would be - 156 mV.

A.4. Discussion

The screening curves obtained for $MgCl_2$ and KCl bear out the observations of Chow and Barber (1980) and Theuvenet *et al* (1984) on the differential effects of mono- and divalent cation salts on 9-aminoacridine release from the surface negative charges. Mg^{2+} was clearly the more efficient cation at displacing the dye.

Comparison of the surface charge density obtained in this study with the data of Theuvenet *et al* (1984) for *S.cerevisiae* is not easy as this latter report was primarily aimed at investigating the linear relationship between the surface potential and the zeta potential and no clear values for q were given. The outer membrane surface of a chloroplast thylakoid bears an excess fixed negative charge density of about 2.5 μC cm^{-2} or approximately one negative charge per 0.064 μm^2 (Mills & Barber, 1978). The value of around - 12 μC cm^{-2} obtained for *C.utilis* cells seems rather high when compared to this other membrane system and is not easy to explain. Interpretation of this value should be limited as, although it was the result of several determinations and whilst the data may represent a reasonably accurate view of the overall negative charge, it is noted that the observed screening curves (Fig. A.3.1.) bear little resemblence to the position and shape of the theoretical profiles which fitted the observed data for chloroplast suspensions (Chow & Barber, 1980). Thus it is the case that whole yeast cells do not easily fit the

theoretical model. Indeed, the closest approximation to the hypothetical screening curves is made when a very high surface charge density is assumed and even so the fit remains unsatisfactory.

One possible explanation for this deviation from the theoretical model may be the presence of negative groups in the yeast cell wall. The model assumes a fixed negative charge uniform over the membrane surface (Barber *et al*, 1977) and if this is complicated by a three-dimensional lattice containing further negative charges at varying distances from the membrane it is clear that the electrical diffuse double layer will be affected. Theuvenet *et al* (1984), using isolated cell walls and protoplasts of *S.cerevisiae*, demonstrated that only 6 % of 9-aminoacridine binding in whole cells could be accounted for by dye binding to whole cells and concluded that the negative charges of the cell wall are probably of minor importance. Furthermore, they suggest that any interactions may be complicated by the negative Donnan potential of the cell wall. The relative importance of the cell wall in *C.utilis* is not known.

One further possible source of error is diffusion of the dye through the plasma membrane or transport *via* the thiamin carrier into the cell (Theuvenet *et al*, 1984). This latter possibility seems unlikely in the present study as cells were extensively starved prior to experimentation.

It seems therefore that 9-aminoacridine is a convenient and simple dye to probe the surface potential of cells. The data shows that in *C.utilis* there exists a detectable negative surface potential which is not of dissimilar magnitude as other membrane systems and it seems likely, on this basis, that transport kinetics in *C.utilis* should not

be affected by the surface potential to any greater extent than in other cell types.

APPENDIX II

PUBLICATIONS

PUBLISHED PAPERS NOT SCANNED AT THE REQUEST OF THE UNIVERSITY.

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